Extensive heterogeneity of the protein composition of *Chlamydia trachomatis* following serial passage in two different cell lines

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To determine if the host-modulated adherence characteristics of the intracellular bacterial pathogen *Chlamydia trachomatis* were due to the acquisition of altered surface-exposed proteins, highly purified chlamydiae grown in two different host cells were analysed. Two serovars, L1 and E, were grown for multiple passages in both HeLa and McCoy host cells. Numerous protein differences in the chlamydial elementary bodies (EB) of each serovar grown in the two different hosts were detected by two-dimensional (2-D) gel electrophoresis and fluorography of radioactively labelled proteins. At least four to six serial passages in the alternative host were necessary before the changes were apparent. Iodination of suspensions of purified chlamydiae and 2-D electrophoresis revealed several surface proteins that were determined by the host cells in which the bacteria had replicated. These iodinated chlamydial proteins were removed by treatment of the iodinated EB with trypsin, indicating their location at the bacterial surface. Two of the major constituents of the outer-membrane complex, the cysteine- and methionine-rich 60 kDa and 40 kDa proteins, remained unchanged in both molecular mass and charge during the host adaptation. Several chlamydial proteins capable of binding iodinated host membrane preparations also exhibited host-dependent alterations. Immunoblotting experiments with a rabbit and a human polyclonal sera indicated that distinct host-specified chlamydial proteins were reactive with the two sera.

Introduction

The identity of ligands mediating the attachment to host cells of *Chlamydia trachomatis*, an obligatory parasitic bacterial pathogen, is not established. Using several lectins as probes to identify the chlamydial ligands responsible for the initial adherence of the elementary bodies (EB) to host cell membrane, we reported that treatment of the host cells with wheat germ agglutinin resulted in a lectin-dose-dependent enhancement of adherence (Bose *et al.*, 1983). In a recent study we noted that the adherence of HeLa- but not McCoy-grown *C. trachomatis* was specifically stimulated when the host cells had been pretreated with the lectin (Bose & Goswami, 1986). In addition, affinity chromatography of ³²P-labelled, highly purified EB on agarose-bound WGA showed that the purified EB of HeLa-grown *C. trachomatis* serovar LGV440 (L1) bound to the lectin gel and could be eluted with buffer containing *N*-acetylglucosamine. After multiple passages of serovar L1 and

another genital serovar, UW31(K), in the alternative host cell McCoy, a mouse fibroblastic cell line, the chlamydiae neither bound to the lectin columns nor showed the lectin-induced enhancement of adherence to the host cells (Bose & Goswami, 1986). Infectivity assays confirmed the affinity chromatography and the adherence data.

During adaptation of yolk-sac-grown isolates of trachoma serovars in McCoy cell cultures, no change was detected in the antigenic properties of the chlamydiae (Gordon *et al.*, 1971). Acquisition of stable infectivity properties by a single passage of egg-grown *Chlamydia psittaci* GPIC in McCoy cell cultures was first noted by Allan & Pearce (1979*a*). After a single passage in cell culture, the progeny acquired enhanced ability to infect McCoy cells without centrifugal deposition of the bacteria onto the monolayer cultures. In addition, neutralization studies revealed that yolk-sac-grown GPIC had an egg-derived antigen that played a role in the unassisted infection of McCoy cells (Allan & Pearce, 1979*b*). Changes in the sedimentation behaviour of the EB of *C. psittaci* 6BC after passage in cell culture (Kordova *et al.*, 1977; Neuman *et al.*, 1980), and in the

Abbreviations: 2-D, two-dimensional; EB, elementary bodies; (M)OMP, (major) outer-membrane protein; TBS, Tris-buffered saline.

lysosomal stability of *C. psittaci* 6BC-infected mouse macrophages (Kordova *et al.*, 1972a) and L cells (Kordova *et al.*, 1972b), have been documented.

We have analysed the protein profiles of highly purified EB of *C. trachomatis* grown in two different host cells, HeLa and McCoy. Distinct alterations in [³⁵S]cysteine/[³⁵S]methionine-labelled proteins and in ¹²⁵I-labelled surface proteins of two serovars, L1 and E, occurred during the growth of *C. trachomatis* in the different cell lines. Chlamydial proteins to which isolated host cell membranes could bind, and the immune reactive proteins were also altered by adaptation in the different host cells.

Some of the data reported here were first presented by Goswami *et al.* (1988) and Vretou *et al.* (1989a).

Methods

Chlamydial growth and purification. *C. trachomatis* serovars LGV440 (L1) and E were grown in monolayer cultures of mycoplasma-free HeLa 229 and McCoy cells (Bose & Goswami, 1986). Infected cells were detached with sterile glass beads, disrupted by sonication, and the EB purified free of contaminating host material and chlamydial reticulate bodies (Vretou *et al.*, 1989b). Mixing experiments had shown that less than 0.02% of host-derived radioactively labelled, hot trichloroacetic acid-precipitable protein co-purified with the EB of *C. trachomatis* (Vretou *et al.*, 1989b), and electron microscopy of the final preparations did not reveal any reticulate bodies, the non-infectious, developmental form of chlamydiae. Detection of mycoplasma was done using an anti-DNA monoclonal antibody from a hybridoma isolated in the laboratory by E. V.

Metabolic labelling of chlamydiae with [³⁵S]methionine plus [³⁵S]cysteine (Trans S). Confluent cultures of HeLa or McCoy cells were infected with chlamydiae, and either 8 or 18 h post-infection with L1 or E, respectively, the medium was replaced with labelling medium that contained 5% of the normal amount of methionine, and emetine, an irreversible inhibitor of eukaryotic protein synthesis, at 1 µg ml⁻¹. After 1 h at 37 °C, 500 µCi [³⁵S]Trans S (*E. coli* hydrolysate containing 70% L-methionine and 15% L-cysteine, 1103 Ci mmol⁻¹, 40.8 TBq mmol⁻¹; ICN Radiochemicals) was added to each 175 cm² flask, and the labelled EB were isolated as usual. Aliquots were stored at -80 °C.

¹²⁵I-labelling of chlamydial EB. Surface-labelling was accomplished with 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen; Pierce). Freshly purified EB was sedimented by centrifugation, the pellet resuspended in HEPES/sucrose/cation buffer (10 mM-HEPES, pH 7.3, 1 mM-MgCl₂, 1.5 mM-CaCl₂, 90 mM-NaCl and 0.2 M-sucrose) and transferred to a microfuge tube coated with Iodogen according to the instructions of the supplier. Carrier-free Na¹²⁵I (100 µCi in 10 µl; ICN Radiochemicals) was added, and held in ice for 10 min. Unreacted isotope was removed by gel filtration of the EB suspension through a column of G-25 Sephadex, equilibrated with phosphate-buffered saline (0.14 M-sodium chloride, 0.01 M-sodium phosphate, pH 7.2) containing 0.5% (w/v) bovine serum albumin and 0.05% NaN₃. Fractions containing the EB were pooled, the chlamydiae concentrated by centrifugation, and aliquots stored at -80 °C. No differences in the polypeptide pattern could be detected between freshly labelled and frozen-thawed samples.

Two-dimensional (2-D) electrophoresis. Isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension was

essentially according to O'Farrell (1975). Samples of radioactively labelled EB retrieved from -80 °C were sedimented by centrifugation and the pellet solubilized (Ames & Nikaido, 1976; Batteiger *et al.*, 1985). After dilution with 2 vols of sample dilution buffer [9.5 M-urea, 5% (v/v) β-mercaptoethanol, 1.6% (v/v) Servalyt ampholytes, pH 3 to 10, 0.4% (v/v) Servalyt, pH 3 to 7, and 8% (v/v) NP-40], the solubilized proteins were loaded on the basic end of the cylindrical focusing gels containing the ampholytes. The electrofocusing was for 16 h at 350 V followed by 1 h at 500 V (6100 V h). For electrophoresis in the second dimension, the cylindrical gels were equilibrated for 15 min in sample buffer (Laemmli, 1970), and secured to the top of a 3% (w/v) polyacrylamide stacking gel. The resolving gel was a 7-20% SDS-polyacrylamide gradient gel. Molecular mass markers were from Bio-Rad. The labelled proteins were detected either by fluorography or autoradiography, depending upon the isotope.

Immunoblotting for the detection of chlamydial proteins. Chlamydial proteins, resolved by either 1- or 2-D-electrophoresis, were transferred (Towbin *et al.*, 1979) to Immobilon P membrane sheets (Millipore) in a Bio-Rad Trans-Blot apparatus at 100 mA constant current for 16 h at 4 °C. Transferred proteins were visualized with Poinceau S, destained, and then blocked for 2 h at 37 °C with 5% non-fat milk in Tris-buffered saline (TBS, 50 mM-Tris/HCl, pH 7.4, 0.9% NaCl) containing 0.05% sodium azide. The blots were incubated with appropriately diluted anti-major outer-membrane protein (MOMP) monoclonal antibody (KB-8), or anti-chlamydial-60 kDa monoclonal antibody (Virostat) or with other sera to detect the immunoreactive chlamydial proteins. After washing the blots three times with TBS containing 0.025% Tween-20, the antibodies bound to the chlamydial proteins were visualized with horseradish peroxidase-conjugated goat anti-human IgG (Dakopatts), anti-mouse (Zymed) or donkey anti-rabbit IgG, (Amersham) and 4-chloro-1-naphthol (Sigma) according to the manufacturers' instructions.

Detection of host cell membrane-binding proteins. Membrane-containing fractions were obtained by two published procedures. Monolayer cultures of HeLa cells were extracted with the non-ionic detergent octyl-β-D-glucopyranoside (Marriott *et al.*, 1987) and the extract was iodinated using Iodobeads (Pierce). The second method used to prepare HeLa membrane was that of Wenman & Meuser (1986). Purified EB of various serovars grown in the different host cells were subjected to 1-D SDS-PAGE, and the chlamydial proteins transferred to Immobilon P membrane sheets (Towbin *et al.*, 1979). After blocking with TBS containing 5% (w/v) non-fat milk, the immobilized chlamydial proteins were incubated at 22 °C for 18 h with the iodinated HeLa membrane fraction. Host cell membrane material bound to the chlamydial proteins was visualized by autoradiography. Identical results were obtained with the membranes prepared by the two different procedures.

Results

2-D analysis of the host-specified ³⁵S-labelled proteins of *C. trachomatis*

C. trachomatis serovars L1 and E were grown in the two different hosts, HeLa and McCoy, for at least 20 serial passages. Chlamydiae were metabolically labelled during intracellular growth in the host cells with a mixture of [³⁵S]cysteine and [³⁵S]methionine (Trans S) in the presence of emetine. The labelled proteins in the purified EB were analysed by 2-D gel electrophoresis and

fluorography. The protein patterns of serovar E grown in HeLa and in McCoy cells (E/HeLa and E/McCoy, respectively) are shown in Figs 1 (a) and (b). The unique, host-specified ^{35}S -labelled proteins detected in these and

similar gels, including those surface-labelled with ^{125}I , are summarized in Table 1. As reported previously (Batteiger *et al.*, 1985) most of the chlamydial proteins are either acidic or neutral. Approximately 120 to 150

Fig. 1. Protein profiles of *C. trachomatis* serovar E, grown for >15 passages in (a) HeLa or (b) McCoy cells. The EB were labelled with ^{35}S -Trans S in the presence of emetine during intracellular growth, purified, and subjected to analysis by 2-D electrophoresis and fluorography as described in Methods. The positions of the MOMP and the 60 kDa OMP2 are indicated. The positions of molecular mass markers (kDa) are also shown.

Table 1. *Unique host-specified proteins of ³⁵S-labelled serovars L1 and E grown in HeLa or McCoy cells*

The data are summarized from the fluorographs shown in Fig. 1, fluorographs of 2-D gels of serovar L1, and autoradiographs of ¹²⁵I-labelled surface proteins of the two serovars. Numbers in parentheses represent distance in cm from the basic end of the gel.

HeLa-specified proteins (kDa)		McCoy-specified proteins (kDa)	
L1	E	L1	E
110* (7.8)	100 (8.6)	112 (6.9)	112 (8.9)
98 (7.8)	98* (7.1)	97* (7.6)	98* (8.5)
95 (7.3)		91 (3.0)	96* (7.1)
90 (6.0)	76* (8.2)	86 (9.8)	83* (6.4)
83* (5.4)		81 (8.2)	75* (6.0)
77 (5.1)		74 (8.6)	65 (6.3)
76 (4.1)	60 (6.8)	73* (5.5)	56 (6.5)
75 (3.8)	56 (4.1)	65* (6.0)	54* (5.2)
75 (5.9)	55* (7.7)	63* (3.9)	51* (6.8)
75 (7.3)	46* (6.0)	61 (6.6)	50 (5.6)
70* (6.8)		54 (2.6)	49 (6.7)
67* (4.1)		46 (3.9)	47 (3.5)
63 (5.8)			46* (5.7)
56* (6.0)			43 (6.0)
	36 (7.3)		42 (2.6)
45 (4.6)	34 (6.8)	45 (5.1)	40 (6.3)
43 (6.0)	32* (8.0)	37 (5.5)	40 (6.6)
35 (2.7)		33 (5.2)	35 (6.2)
34 (6.3)		32* (4.4)	33* (7.0)
32* (6.3)		31* (5.2)	32 (7.5)
			22* (4.8)
26* (7.6)	26* (8.2)	29 (6.5)	21* (6.4)
24* (3.3)		24 (7.6)	

* ¹²⁵I-labelled surface proteins.

labelled spots were detected in these and other gels. An immunoblot of a similar gel with an anti-60 kDa monoclonal antibody detected the OMP2 (Allen & Stephens, 1989), a cysteine- and methionine-rich protein, at the cathodic end, as had been noted for non-LGV serovars (Batteiger *et al.*, 1985). Eleven unique HeLa-specific proteins and 22 McCoy-specific ones were detected in these and other gels of *C. trachomatis* serovar E (Table 1). It should be noted that no host-dependent change was detected in the molecular mass or the pI of the MOMP or of the 60 kDa OMP2 protein and these two proteins served as highly reproducible internal markers to align the fluorographs. There were several proteins of identical molecular mass but differing in pI among the host-specified proteins of serovar E (Table 1). Identical data were obtained from replicate experiments.

Numerous unique host-specified ³⁵S-labelled proteins of an LGV serovar (L1) grown for multiple passages in HeLa and in McCoy cells were also detected on

fluorographs of the 2-D gels and their properties have been summarized in Table 1. Twenty-one HeLa- and 19 McCoy-specific proteins of serovar L1 were detected. Some of the proteins migrated to the same molecular mass regions but had different pI. No change in either the molecular mass or the pI of the MOMP or OMP2 was seen in the EB of serovar L1 due to host passage, similar to the situation with serovar E. The 45 kDa protein of L1/McCoy had a lower pI than the identical protein from L1/HeLa (Table 1). The 32 kDa protein detected in the EB of L1/McCoy had a different pI relative to the identical protein in the L1/HeLa (Table 1). The 2-D gel analysis of HeLa- or McCoy-grown serovar L1 has been done three times with identical results, ruling out the possibility of variations in the IEF gels contributing to the changes detected.

The host-specific proteins that were detected after 2-D electrophoresis of the purified EB of either serovar E or L1 after growth adaptation in HeLa or McCoy cell lines were apparent only after five to six serial passages in the respective host (data not shown). The appearance and disappearance of the unique chlamydial proteins characteristic of the host cell were reversible.

Host-specified surface proteins of chlamydiae

Serovars L1 and E were grown for >20 passages in HeLa and in McCoy cells. Purified EB were iodinated as described in Methods. After 2-D electrophoresis and autoradiography, 23 spots from the purified EB of L1/HeLa, and 19 from L1/McCoy could be detected (Fig. 2a). Similar analysis of the ¹²⁵I-labelled surface proteins of *C. trachomatis* serovar E revealed 15 spots from E/HeLa, and 19 from E/McCoy (Fig. 2b). The unique host-specific proteins detected in the autoradiographs are marked with an asterisk in Table 1. Among these were the following: eight HeLa-specific and four McCoy-specific proteins in the EB of serovar L1; seven HeLa-specific and 11 McCoy-specific ¹²⁵I-labelled proteins in serovar E. When the iodinated EB suspension was incubated with trypsin (1 µg per 10 µg chlamydial protein, 2 h at 37 °C), none of the iodinated spots could be detected by 2-D analysis and autoradiography.

Host-modulated membrane-binding proteins

HeLa- and McCoy-adapted serovar E and L1 proteins were separated by 1-D electrophoresis, and transferred to Immobilon P membranes by the method of Towbin *et al.* (1979). Iodinated HeLa membrane was used to probe the membrane-binding chlamydial proteins as described in Methods. Protein bands additional to the 32 and 18 kDa proteins (Hackstadt, 1986; Wenman & Meuser, 1986) were detected on the Immobilon P membranes, pre-

Fig. 2. Composites of paired autoradiographs of the surface-iodinated protein profiles obtained by 2-D electrophoresis of *C. trachomatis* serovars L1 (a) and E (b) grown for multiple passages in HeLa and in McCoy cells. The purified EB were labelled extrinsically with ^{125}I as described in Methods, and subjected to 2-D electrophoresis and autoradiography. ●, Proteins common to both HeLa- and McCoy-grown chlamydiae; ○, proteins unique to HeLa-grown chlamydiae; □, proteins unique to McCoy-grown chlamydiae. M is the location of the MOMP of both samples. Positions of molecular mass markers are shown.

sumably due to a superior transfer of the chlamydial proteins from the gels to the Immobilon P membranes and by the buffer system utilized for the transfer (Towbin *et al.*, 1979). Both types of HeLa membranes bound to essentially identical chlamydial proteins of the two serovars. The results are summarized in Table 2. HeLa- as well as McCoy-specific proteins of molecular mass 70, 58, 40, 32 and 18 kDa in serovars L1 as well as E consistently exhibited the ability to bind isolated iodinated host cell membrane. The 58 kDa protein was as capable of binding the HeLa membrane preparations as were the 32 and 18 kDa chlamydial proteins. It was ascertained to be the cysteine-rich OMP2 by immunoblotting with an anti-60 kDa monoclonal antibody. The

Table 2. Membrane-binding proteins of *Chlamydia trachomatis* serovars E and L1: effect of adaptation in HeLa or McCoy cells

Membrane-containing material was prepared from monolayer cultures of HeLa cells (Wenman & Meuser, 1986), and iodinated using Iodobeads. The chlamydial proteins were resolved by 1-D electrophoresis, electroblotted to an Immobilon P membrane and probed with iodinated HeLa membrane as described in Methods.

Molecular mass range (kDa)	Membrane-binding proteins (kDa)			
	L1		E	
	HeLa	McCoy	HeLa	McCoy
75–50	70	70	70	70
	58	58	58	58
50–39		50*	51*	
	45*			
	43*	44*	43*	
39–27	40	40	40	40
		39*		
				37*
27–17	32	32	32	32
	18	18	18	18

* Unique proteins.

Table 3. Host-passage-dependent unique antigenic determinants of serovar E

Proteins of *C. trachomatis* serovar E that had been passaged > 10 times in HeLa and in McCoy cells were separated by 1-D gel electrophoresis, electroblotted on to a nitrocellulose membrane, and incubated with appropriately diluted sera from a rabbit immunized with *C. trachomatis* serovar L2/McCoy, and from a patient who had acquired infection with *C. trachomatis* L2 through aerosol inhalation during sonication of infected HeLa cells.

Rabbit antiserum		Human antiserum	
E/HeLa	E/McCoy	E/HeLa	E/McCoy
67	67	67	67
62*	–	62*	–
60	60	60	60
58	58	58	58
–	–	56*	–
–	–	–	55*
54*	–	54*	–
47	47	47	47
MOMP	MOMP	MOMP	MOMP

* Unique proteins.

unique HeLa-specific proteins had molecular masses of 45 and 43 kDa in serovar L1 and of 51 and 43 kDa in serovar E (Table 2). The McCoy-specific chlamydial proteins that bound iodinated membrane fragments of HeLa cells were the following: for serovar L1, 50, 44 and 39 kDa; and for serovar E, only one protein of 37 kDa.

Host-modulated chlamydial antigens

The proteins of E/HeLa and E/McCoy were analysed by immunoblotting using a polyclonal serum from a rabbit immunized with *C. trachomatis* L2/McCoy, and a serum from a laboratory member who had accidentally acquired a chlamydial infection by the inhalation of aerosol during sonication of L2-infected HeLa cells. Both sera reacted very strongly with the 60 kDa protein and the MOMP of E/HeLa and E/McCoy. Both sera recognized two unique E/HeLa proteins of 62 and 54 kDa. The human serum recognized an additional unique 56 kDa protein in the EB of E/HeLa, and another one in E/McCoy of 55 kDa (Table 3).

Discussion

This study was initiated to determine if the host-passage-dependent altered adherence of *C. trachomatis* to mammalian cells (Bose & Goswami, 1986) resulted from the acquisition of host-specified proteins that mediated this step. Data presented here indicate that numerous protein differences develop between the same serovar of chlamydiae after growth in different hosts. These unique host-specified chlamydial proteins include several that are surface-exposed (Figs 1 and 2, Table 1). Since these alterations become detectable only after more than four passages in the two hosts and in a reversible manner (data not shown), host protein contamination in the EB preparations is ruled out as the probable reason for these differences. Iodination of the purified EB resulted in labelling of only the surface proteins, since treatment of the iodinated EB suspension with trypsin removed all the labelled proteins. We were unable to detect iodinated 18 kDa protein in serovars L1 and E, regardless of their passage history (Fig. 2, Table 1). Also noteworthy are the host-passage-dependent changes in the 32 kDa protein labelled with ¹²⁵I in serovars E and L1 grown in HeLa or in McCoy cells (Table 1).

At least two proteins have been isolated from several serovars of *C. trachomatis* that bind Triton X-100-solubilized membrane-containing material from HeLa cells (Hackstadt, 1986). Similar HeLa-membrane-binding chlamydial proteins of 18 and 32 kDa were also found in serovars J and L2 (Wenman & Meuser, 1986). In both studies the chlamydiae had been propagated in HeLa cells. Our membrane-blotting experiments detected several additional and host-specific chlamydial proteins capable of binding membrane preparations from HeLa cells (Table 2). When the electrophoretic transfers were done in the phosphate buffer system used by Hackstadt (1986) and Wenman & Meuser (1986), only the 32 and 18 kDa proteins exhibited membrane-binding

activity (data not shown). Experiments are in progress to determine which, if any, of these multiple chlamydial proteins mediate the adherence step in the initiation of infection.

Immunoblotting experiments with serovar E grown in HeLa or in McCoy cells, using as probes serum from an immunized rabbit, and from an individual who was accidentally infected with *C. trachomatis*, have shown that the immune response includes common as well as host-specific antigens. Among the many chlamydial proteins that reacted with the two polyclonal sera, proteins of 62, 56, and 54 kDa were present only in the EB of E/HeLa (Table 3). Only a single unique E/McCoy-specific protein (55 kDa) was detected using the human serum. It is noteworthy that the rabbit was immunized with L2/McCoy, while the patient had been infected with L2/HeLa.

Analyses of the whole EB lysates, the surface-exposed proteins, the membrane-binding proteins and the response to the polyclonal sera suggest that with the adaptation of chlamydial serovars to growth in the different hosts, numerous proteins undergo modulation of expression determined by the host cell. Some of these host-specified proteins are surface-exposed, and some appear to be unique antigenic determinants. Interestingly, the two major constituents of the outer membrane of this intracellular bacterium, the MOMP and the OMP2 proteins, remain conserved in both molecular mass and pI during the host adaptation, and indeed serve as internal markers for 2-D analyses. It seems rather tenuous to consider these numerous chlamydial proteins to be host-modulated 'stress proteins'. Nevertheless, it is possible that chlamydial growth in the different host cells that are routinely used in laboratories induces host-modulated expression of proteins whose functions remain unknown.

We had speculated that this intracellular pathogen might acquire surface antigens that enable it to escape the host's immune surveillance system (Bose & Goswami, 1986). The surface protein labelling and the immunoblotting data presented here reinforce this hypothesis, implying that host-specific proteins may be involved in the immune response mechanism. While additional evidence must be obtained to show that true antigenic variation occurs in the proteins involved in immunogenicity following chlamydial infection, it seems reasonable to us that this obligately parasitic bacterium mimics other bacterial and viral pathogens which manage to survive in a hostile milieu.

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