Phage-display antibody detection of *Chlamydia trachomatis*-associated antigens

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A phage-displayed human single-chain Fv antibody library (6.7×10^9 members) was used to select probes specific to components associated with the surface of *Chlamydia trachomatis* elementary bodies (EBs). Each of 15 antibodies was characterized by ELISA, dot-blot, immunoblot and immunocytochemistry, resulting in the identification of several new chlamydial components associated with the surface of EBs. In addition, six antibodies were specific for host-cell components associated with the surface of EBs. While phage display has been used effectively to produce specific antibodies for purified components, these data show that this technology is suitable for selection of specific probes from complex antigens such as the surface of a microbial pathogen.

Keywords: intracellular bacteria, outer-membrane proteins, recombinant antibody

INTRODUCTION

There is a need to develop clonal reagents for diagnostic assays, purification and molecular characterization of molecules on the surfaces of microbial pathogens. Filamentous bacteriophages have been used to display single-chain variable fragment (scFv) and Fab antibody fragments by fusion to the phage minor coat protein pIII (Hoogenboom *et al.*, 1991; McCafferty *et al.*, 1990). Display of large and diverse antibody fragment gene repertoires on phages permits the direct selection of monoclonal antibodies using antigen-affinity chromatography without the need for immunization (Marks *et al.*, 1991). Advantages of phage-display antibody technology include the following: (i) selection of antibodies from a phage library is unbiased by the immunogenicity and less dependent on quantitative abundance of the target ligands (Schier *et al.*, 1996); (ii) antibodies are produced in DNA-encoded plasmids that are readily cloned and modified; and (iii) antibodies can be produced in large quantities from *Escherichia coli* without the use of animals. For this study, a 6.7×10^9 -member scFv phage antibody library (Sheets *et al.*, 1998) was used to probe the surface of *Chlamydia trachomatis* elementary bodies (EBs) to identify novel surface components.

Chlamydia spp. are obligate intracellular bacteria that cause disease in humans and other animals. *C. trachomatis* causes a spectrum of diseases in humans, including trachoma (a leading cause of blindness) and sexually transmitted infections that often result in severe sequelae in women such as infertility, ectopic pregnancy and chronic pelvic pain (Schachter, 1999). *Chlamydia* spp. have a developmental cycle that begins with the EB, the infectious, metabolically inactive, osmotically stable extracellular form that enters the host cell and remains within a specialized host-cell vacuole, referred to as an inclusion. The EB outer membrane is extremely stable and contains multiple disulfide-bonded outer-membrane proteins (Hatch *et al.*, 1984). Because *Chlamydia* spp. are obligate intracellular pathogens, the macromolecular surface components of EBs are of particular importance for their unique biology. However, there is a narrow understanding of the macromolecular composition of chlamydial outer membranes, and few surface-exposed components have been defined at a molecular level. Even though extensive research has been conducted, all of the potential mechanisms for chlamydial attachment and infectivity have yet to be elucidated (Stephens, 1999). Adhesion or invasion ligand candidates include heparan-sulfate-like molecules (Zhang & Stephens, 1992), the major outer-membrane protein (MOMP) (Su *et al.*, 1988), glycosylated MOMP (Swanson & Kuo, 1994), 18 kDa and 32 kDa glycoproteins (Swanson & Kuo, 1990) and exoglycolipid (Stuart *et al.*, 1991). Of these, only a few have been

Abbreviations: EB, elementary body; HRP, horseradish peroxidase; scFv, single-chain variable fragment.

demonstrated as surface exposed and studied at a molecular level.

Investigations to define the composition of the outer membrane have been biased by the procedures available to extract the outer membrane as well as limited by availability of probing reagents, primarily monoclonal antibodies. Monoclonal antibodies are typically dependent upon selection to dominant immunogens. The information inferred by electron microscopy of chlamydial EBs shows complex surface structures (Matsumoto, 1981), and proteins identified from the genome sequence of *C. trachomatis* predicted to be localized to the outer membrane (Stephens *et al.*, 1998) suggest a more diverse composition than has been elucidated thus far.

In this study, a panel of scFv phage antibodies to EB surface molecules was generated by panning the phage antibody library on *C. trachomatis* EBs adsorbed to polystyrene tubes. The scFv antibodies were characterized for reactivity by ELISA, dot-blot, immunoblot and immunocytochemistry. Several chlamydial surface proteins probably not previously identified by other methods were identified by specific scFv antibodies. In addition, scFv antibodies specific for host-cell antigens associated with the surface of EBs were selected that would not otherwise have been identified from the *C. trachomatis* genome sequence (Stephens *et al.*, 1998).

METHODS

Bacterial strains. *Chlamydia trachomatis* serovar L2 (L2/434/Bu) EB and *Chlamydia psittaci* AP-1 EB were purified from L929 cells grown in suspension culture. *C. trachomatis* serovar D (D/UW-3/Cx) and serovar L2 EB were purified from both HeLa229 cells and L929 cells grown in monolayer culture. The L929 and HeLa229 cells were from C. C. Kuo, the University of Washington, Seattle. EB preparations used for scFv phage antibody selection were purified using 30% and 30–44% discontinuous Renografin gradients (E. R. Squibb and Sons) (Koehler *et al.*, 1990). EBs used for subsequent analysis and characterization were purified using 30–44–55% discontinuous Renografin gradients. For phage rescue and scFv phage antibody expression, *E. coli* TG-1 cells were used (Marks *et al.*, 1991).

Selection of scFv antibodies specific for EBs. The selection for scFv antibodies that bound chlamydial EBs included three discrete steps: selection of bound phage particles, recovery of phagemids encoding scFv antibody, and production of phage for additional rounds of selection. For the selection procedure, the method of Marks *et al.* (1991) was used with the exceptions noted below. Briefly, immunotubes (Nunc Maxisorp) were coated overnight with a 1 ml suspension of *C. trachomatis* (serovar L2) EBs (0.1 mg ml⁻¹ in PBS) at 4 °C. The tubes were washed, blocked and 10¹² phage particles from a phage library containing 6.7 × 10⁹ members (Sheets *et al.*, 1998) were added and allowed to bind for 1 h at room temperature. The nonadherent phage particles were removed by washing. Bound phage were eluted using three separate methods from three EB-coated immunotubes in an effort to obtain a variety of scFv antibodies with different specificities. First, 1 ml 100 mM triethylamine was added for 10 min then neutralized with 0.5 ml 1 M Tris/HCl pH 7.4. Second, phage were eluted by

shifting the temperature to 43 °C for 15 min. Third, 0.2 units heparitinase (Seikagaku America) in 250 µl PBS was used to elute phage for 15 min at 37 °C.

Eluted phage were used to infect *E. coli* TG-1 which were plated on 2 × TYE plates (Miller, 1972) containing 100 µg ampicillin ml⁻¹ and 1% glucose (2 × TYE/amp/glu). Following overnight incubation, the bacteria were scraped from the plates into 2 × TYE medium containing 2 × TYE/amp/glu and phage antibodies were prepared by rescue with VCSM13 helper phage (Stratagene) as previously described (Marks *et al.*, 1991). After overnight growth, the sample was centrifuged and phage were concentrated from the supernatant by precipitation with polyethylene glycol and filtered through a 0.45 µm filter. The concentrated phage (1.0 × 10¹¹ particles ml⁻¹) were used for the next round of selection on EB. A total of three rounds of selection was performed.

After the third round of selection, soluble scFv antibodies were expressed from individual *E. coli* colonies as described by Marks *et al.* (1991). Briefly, scFv phage-displayed antibodies were screened for binding to EBs by ELISA (see below). The number of unique ELISA-positive clones was determined by *Bst*NI fingerprinting (Clackson *et al.*, 1991). scFv genes were amplified from single *E. coli* colonies with the primers LMB3 (5'-CAGGAAACAGCTATGAC-3') and fd-SEQ1 (5'-GAATTTTCTGTATGAGG-3') followed by digestion of the PCR product with *Bst*NI (Marks *et al.*, 1991). Clones were classified according to the fingerprint pattern and one clone of each fingerprint was selected for further characterization.

Isolation of soluble scFv antibodies. Soluble scFv antibodies were prepared for the antibody-binding studies. scFv genes were initially contained in the vector pHEN1 (Schier *et al.*, 1995) for secretion as scFv antibodies with a C-terminal c-Myc-epitope tag (Evan *et al.*, 1985). Native scFv antibodies were expressed from *E. coli* TG-1 as previously described by induction with IPTG (0.5 mM) and growth at 25 °C for 5 h (Marks *et al.*, 1991). scFv antibodies were harvested from the bacterial periplasm by osmotic shock (Breitling *et al.*, 1991). Because of cross-reactivity of the anti-c-Myc antibody 9E10 (Santa Cruz Biotechnology) with several chlamydial proteins, scFv genes were also subcloned into the vector pCANTAB 5E (Amersham Pharmacia Biotech). This vector fused a C-terminal E-epitope tag to the scFv antibodies which could be detected with anti-E monoclonal antibody (Amersham Pharmacia Biotech). Conservation of the scFv gene was confirmed by repetition of the fingerprinting assay using the primers LMB3 and LMB2 (5'-GTAAACGACGGCCAGT-3').

Binding assays. scFv antibodies were normalized by examination of relative amounts of scFv antibody in each individual periplasmic preparation. The relative amount of scFv antibody was quantified by nitrocellulose spot assay as observed by intensity of the dots. Briefly, 50 µl of each scFv periplasmic preparation was spotted onto nitrocellulose membranes. Membranes were blocked with 3% BSA in PBS for 1 h at room temperature. scFv antibodies were detected using either 9E10 or anti-E-tag antibody followed by the addition of horseradish peroxidase (HRP)-conjugated anti-mouse Fc antibody (Sigma) and 4-chloro-1-naphthol substrate (Fisher Biotech).

ELISA. scFv antibodies were screened for binding to chlamydial EBs by ELISA. Briefly, 96-well microtitre plates (Immulon 2, Dynex Technologies) were coated overnight with 50 µl 0.1 mg ml⁻¹ purified EBs in PBS. Plates were blocked with 5% skim milk in PBS (MPBS) and 50 µl of *E. coli* supernatant containing scFv antibodies was added. scFv antibody binding was

detected using either 9E10 or anti-E antibody, HRP-conjugated anti-mouse Fc antibody and OPD substrate (Dako Corporation).

Immunoblot. Immunoblot assays were used to determine if scFv antibodies bound identifiable bands in lysates of EBs and/or host cells. Fifty micrograms of purified EBs, HeLa229 or L929 cells were tested per lane and immune reactions were determined using either c-Myc-tag or E-tag scFv antibodies, their corresponding anti-tag antibodies and anti-Fc peroxidase conjugate. Ultimately the anti-E antibody was found to react to EBs by immunoblot; therefore the anti-E antibody was preabsorbed with a final concentration of $10 \mu\text{g ml}^{-1}$ of lysed (20 mM DTT, boiled) purified EBs. Reactivity was detected using enhanced chemoluminescence (ECL, Amersham Pharmacia Biotech). EBs were run on 10% SDS-PAGE gels for all immunoblots and transferred to nitrocellulose.

Immunocytochemistry. Immunocytochemistry of *Chlamydia*-infected and uninfected HeLa229 cells and L929 cells was performed using confluent host cell monolayers on 12 mm glass coverslips in 24-well tissue culture plates. Samples of 200 μl containing $\sim 8 \times 10^6$ inclusion-forming units ml^{-1} were used to infect monolayers (multiplicity of infection of 4). The infected cells were incubated for 48 h at 37°C , washed with PBS and fixed with 100% methanol for 10 min; subsequent washes were performed with PBST (0.05% Tween 20). Each scFv antibody was incubated on the fixed cells for 1 h at room temperature and probed with anti-E antibody in 2% MPBS. Immune reactions were detected using FITC-conjugated anti-mouse Fc sera (Zymed) diluted in 2% MPBS for 1 h at room temperature. The cells were counterstained with Evans Blue and visualized using a fluorescent microscope. For immunofluorescence assays involving unfixed cells, scFv antibodies were applied directly to the live cells for 1 h at 37°C , after which the cells were methanol fixed. Subsequent detection incubations were as stated above.

EB dot-blot assay for surface-accessible antigens. The dot-blot assay developed by Zhang *et al.* (1987) permits the specific detection of surface-localized antigens. The assay was performed as previously described (Zhang *et al.*, 1987) with modifications noted. Briefly, 50 μl samples of purified serovar L2 EBs ($5 \mu\text{g ml}^{-1}$) were applied to nitrocellulose in a dot-blot apparatus (Gibco-BRL) by gravity filtration for 10 min then vacuum was applied for 10 s. The nitrocellulose was then blocked with 3% BSA in PBS and washed. Fifty microlitres of normalized amounts of scFv antibody were added to each well and left for 1 h at room temperature. Anti-E antibody and anti-mouse Fc-peroxidase conjugate were used to probe for bound scFv antibodies. Reactivity was detected by ECL. For some dot-blot assessments EBs were digested using 50, 100 or 500 μg proteinase K ml^{-1} for 20 min at 37°C prior to conducting the assay. To inhibit activity of proteinase K, PMSF at a concentration of 1 mM was added simultaneously with the proteinase K to the solution containing EBs. To ensure that intact EBs were predominant in the dot-blot assay, EBs were subjected to 2% SDS to lyse the organisms and expose intracellular antigens.

RESULTS

Selection of *C. trachomatis*-specific phage-display antibodies

Phage-display antibody technology was utilized to select probes to surface components on *C. trachomatis* EBs. Phages specific for surface components on EBs were

affinity-selected from a large phage-display antibody library (Sheets *et al.*, 1998). Enrichment for phage binding is measured by the number of bound phage recovered after each elution step. In order to isolate a broad spectrum of phage antibodies with different specificities, three elution procedures were used. Each set of three rounds of selection included elution by mild heat treatment, heparitinase digestion, or the conventional alkaline treatment using triethylamine (Marks *et al.*, 1991). Three rounds of selection resulted in 1000-fold enrichment of recovered phage-infected *E. coli* for each method, from approximately 10^4 ml^{-1} after round 1, to 10^5 ml^{-1} after round 2, to 10^7 ml^{-1} after round 3.

A total of 368 scFv clones from the enrichment/selection methods was individually assessed for binding to purified EBs by ELISA. ELISA-positive reactions were considered arbitrarily to be twice the absorbance of the background conjugate control. This limit was selected so scFv antibodies that might be binding molecules of low abundance on EB would not be eliminated. By this criterion 132 scFv clones were considered positive for binding EBs. The nitrocellulose spot assay was used to determine which of the clones were efficiently expressing scFv antibodies. Forty-seven of the scFv clones produced weak reactions in the nitrocellulose spot assay and were eliminated. Next, restriction endonuclease fingerprint analysis was used to evaluate the diversity of scFv clones. By this assay 61 scFv clones were likely duplicates and not evaluated further. Nine clones were lost during these steps due to a variety of causes such as loss of clone viability or scFv antibody expression. A total of 15 scFv antibodies that met the screening criterion was used in the subsequent analyses.

Species specificity of scFv antibodies

Although the scFv phage antibody library was selected using *C. trachomatis* serovar L2 EBs, it could be expected that some scFv antibodies may not only recognize serovar-specific antigens, but species- and genus-specific antigens as well. This spectrum of reactivity has been observed previously using monoclonal antibodies (Stephens *et al.*, 1982). Soluble scFv antibodies were prepared and relative quantities normalized using the nitrocellulose spot assay. scFv antibody specificity was assayed by ELISA, immunoblot, and immunofluorescence testing the reactivity to EBs prepared from *C. trachomatis* LGV biovar (serovar L2) and a trachoma biovar (serovar D) and *C. psittaci* (Cp) (AP-1, avian) (Table 1). Ten individual scFv antibodies bound all three strains of *Chlamydia* by at least one of the assays (ELISA, immunoblot or immunofluorescence), suggesting that the scFv antibodies recognize a genus-specific antigen. Four scFv antibodies bound only to EBs from *C. trachomatis* serovars L2 and D, suggesting an antigen shared at the species level. One scFv antibody (3H1-F2) was shown ultimately not to bind EBs by ELISA, immunoblot and immunofluorescence staining, although by the nitrocellulose spot assay large amounts of scFv antibodies were produced.

Table 1. Summary of each scFv antibody and its corresponding binding characteristics to two *C. trachomatis* serovars (L2 and D), *C. psittaci* (Cp), and murine L929 and human HeLa cells

Clone	ELISA			Immunoblot*				Fluorescence staining				
	L2	D	Cp	L2	D	Cp	L929	Inclusion			Uninfected	
								L2	D	Cp	L929	HeLa
E-D4	++	++	++	+	—	+	+	+	+	+	+	+
3H1-B1	+	+	+	55	—	55	—	+	+	—	—	—
3H1-D2	±	±	±	42, 230	42, 230	42, 230	230†	—	—	—	+	—
3H1-E10	±	±	±	230	230	230	230†	—	—	—	+	—
E-A11	+	+	+	165	75	110	—	+	—	—	—	—
E-B11	++	++	++	165	110	110	+	+	+	—	±	±
H-E11	++	++	+	165	110, 75	110	—	+	+	—	—	—
3H1-A5	+	±	+	73	73	73	+	—	—	—	+	+
E-B1	—	—	—	—	—	—	—	+	+	+	—	—
3H2-D6	+	+	+	—	—	—	—	—	—	—	—	—
2H-G2	++	++	—	69	69	—	—	+	+	—	—	—
H-B6	++	++	—	69	69	—	—	+	+	—	—	—
H-B11	++	++	—	69	69	—	—	+	+	—	—	—
2H-B9	++	++	±	71	71	—	+	+	+	—	±	±
3H1-F2	—	—	—	—	—	—	230	—	—	—	+	+

* Numbers represent estimated molecular masses (kDa); + indicates that the scFv antibody recognized multiple bands.

† The scFv antibody recognized a band only in L929 cells not in HeLa229 cells.

Macromolecular identification of *Chlamydia*-specific scFv-antibody-recognized antigens

Macromolecular antigen identification was assayed by immunoblot to determine the approximate molecular masses of scFv-antibody-bound antigens. scFv antibodies E-B1, 3H2-D6 and 3H1-F2 did not show reactivity by immunoblot to purified chlamydial EBs. scFv antibody E-D4 bound multiple components by immunoblot, precluding a specific assignment of immunoreactivity (data not shown). Eleven scFv antibodies showed strong reactivity to specific molecules with purified *C. trachomatis* serovar L2 EB lysates by immunoblot, and the molecular mass was estimated for each (Table 1). Three of these scFv antibodies (E-B11, 3H1-A5 and 2H-B9) recognized an exclusive antigen in purified serovar L2 EB lysates, but they non-specifically detected other multiple components in uninfected host cells. These scFv antibodies were considered *Chlamydia*-reactive because of the relative strength of reactivity and the fact that the estimated molecular mass of the antigen reactive in *Chlamydia* EB lysates was not the same as any component recognized in uninfected host cell lysates (data not shown).

Because many of the scFv antibodies reacted to specific antigens in purified serovar L2 EB lysates and had genus-specific or species-specific reactivity as determined by ELISA, each scFv was used in immunoblot assays using lysates from *C. trachomatis* serovar D and *C. psittaci*. For the scFv antibodies that recognized serovar D and/or *C. psittaci* antigens, the estimated molecular masses were the same as those observed in serovar L2,

except for E-A11, E-B11 and H-E11 (data not shown). Each of these latter three scFv antibodies bound an antigen of ~165 kDa in serovar L2 and one of ~110 kDa in *C. psittaci*, but in serovar D they were reactive to a ~75 kDa antigen and a ~110 kDa antigen (Table 1). scFv antibodies 2H-G2, H-B6 and H-B11 bound only a ~69 kDa doublet antigen in both of the *C. trachomatis* biovars. 3H1-B1 recognized a ~55 kDa component only in serovar L2 and *C. psittaci* but not in serovar D. An example of each antigen-specific reactivity is shown in Fig. 1.

Cellular localization of scFv antibody-reactive antigens

Immunofluorescence staining of uninfected human HeLa229 cells and murine L929 cells, and both cell lines infected with *C. trachomatis* serovars L2 and D and *C. psittaci*, was used to test both the specificity of binding and the subcellular location of the antigens recognized by scFv antibodies. HeLa229 cells were chosen for initial analysis as it was possible that L929-specific scFv antibodies were selected since the initial serovar L2 EBs were purified from L929 cells. There were two patterns of staining by the scFv antibodies specific for chlamydial inclusions. One group of scFv antibodies (3H1-B1, E-A11, H-E11 and E-B1) showed a punctate staining pattern limited to inclusions consistent with the phenotypes of EBs and RBs (reticulate bodies) (Fig. 2a); E-B11 and 2H-B9 also displayed punctate staining within the inclusion but additionally stained infected and uninfected host cells with a relatively weak and diffuse

Fig. 1. Immunoblot analysis of purified serovar L2 EBs probed with individual scFv antibodies. Equivalent amounts of purified serovar L2 EBs were loaded in each lane, probed with each scFv antibody and detected with mouse anti-E antibody and anti-mouse Fc antibody. Individual scFv clone numbers are indicated above each lane. Calculated estimates of molecular masses (kDa) are shown on the left.

Fig. 2. Immunofluorescence microscopy of *C. trachomatis* serovar L2-infected host cells at 48 h post-infection using selected scFv antibodies. (a–d) Infected HeLa 229 cells. Each panel was probed with individual scFv antibodies considered to be representative of unique patterns of staining, detected with anti-E antibody, FITC conjugated anti-mouse Fc antibody and counterstained with Evans Blue. scFv antibody E-B1 (a) demonstrated punctate staining, 2H-G2 (b) represented larger structure staining patterns. Both 3H1-A5 (c) and 3H1-F2 (d) recognize cytoskeletal-like components. (e, f) scFv antibody 3H1-E10 was used to stain uninfected L929 cells (e) and HeLa 229 cells (f).

staining character (data not shown). The second staining pattern observed for another group of antibodies (2H-G2, H-B6 and H-B11) was also limited to the inclusion but revealed larger structures within the inclusions (Fig. 2b).

Four scFv antibodies each bound antigens in infected cells, but the staining was not limited to, and apparently excluded from, the chlamydial inclusion vacuole. These scFv antibodies also bound uninfected host cells and unique patterns of staining with respect to each other were observed. scFv antibodies 3H1-A5 (Fig. 2c) and 3H1-F2 (Fig. 2d) stained infected cells with a pattern reminiscent of cytoskeletal structures yet were dissimilar from each other. These two antibodies were also reactive to antigens in uninfected HeLa229 and murine L929 cells (data not shown). Another host cell staining pattern was observed for the scFv antibodies 3H1-D2 and 3H1-E10. Although the staining was excluded from the inclusion in infected cells, it apparently stained between host cells or associated with cellular projections similar to the pattern seen in uninfected L929 cells (Fig. 2e). Unlike the scFv antibodies 3H1-A5 and 3H1-F2, the staining exhibited by 3H1-D2 and 3H1-E10 was limited to L929 cells and not observed in HeLa229 cells (Fig. 2f). This was the same specificity as observed by immunoblot (see below).

Despite the observation that 3H1-A5, 3H1-E10 and 3H1-D2 stained uninfected host cells by immunofluorescence, consistent with the host cell immunoblot data, the scFv antibodies nevertheless bound to purified EBs by ELISA, immunoblot and dot-blot (see below). These data suggest that host-cell antigens not associated with intracellular chlamydiae become associated with EBs following lysis and exit from the host cell.

Surface localization of scFv-antibody-recognized antigens

Due to the nature of the selection procedures, elution of phage-displayed antibodies from EBs would be expected to generate probes for chlamydial surface components. Surface localization of the scFv-antibody-recognized antigens was tested using the dot-blot assay to detect chlamydial surface antigens as described by Zhang *et al.* (1987). scFv antibody preparations were adjusted for approximately equal amounts of scFv antibody and used in the dot-blot assay probing purified *C. trachomatis* EBs. Although some of the scFv antibody reactivities were relatively weak, each of the scFv antibodies bound EBs by dot-blot (Fig. 3). To ensure that EBs were intact during the dot-blot assay, EBs were subjected to lysis by SDS treatment then probed with the anti-E-tag antibody that is cross-reactive to an internal chlamydial protein as well as all scFv antibodies (data not shown). Following lysis of EBs, anti-E-tag antibodies showed increases in dot intensity, indicating that EB lysis occurred without overall loss of antigen quantity and that internal antigens became accessible to antibody binding by EB lysis (Fig. 3). Additionally, scFv antibodies 3H1-B1, 3H2-D6 and E-A11 also showed an increased binding upon EB lysis,

Fig. 3. Dot-blot assay using individual scFv antibodies to probe purified serovar L2 EBs and demonstrate proteinase K sensitivity of the epitopes recognized by scFv antibodies. Columns labelled scFv demonstrate the amount of each scFv antibody used to detect the purified EBs; relative amounts of individual scFv antibodies are indicated by dot intensity. The column labelled EB represents serovar L2 EB probed with each scFv antibody, anti-E antibody and HRP-conjugated anti-mouse Fc antibodies, detected by ECL. The columns labelled 5 and 100 indicate scFv antibody binding to EBs digested with 5 or 100 µg proteinase K ml⁻¹. The column labelled PMSF indicates EBs digested with 100 µg proteinase K ml⁻¹ with PMSF added. The experiment in which EBs were pretreated with SDS to facilitate lysis of intact organisms is shown in a separate panel (labelled SDS); the blot was probed with anti-E antibody.

whereas the remaining scFv antibodies revealed dots of equal intensity regardless of whether the EBs were lysed or not (data not shown).

Proteinase K digestion was used to assess the composition of the scFv-antibody-recognized antigens. Concentrations of 5, 100 and 500 µg proteinase K ml⁻¹ were used to assess antigen sensitivity. Most of the scFv-antibody-recognized antigens were sensitive at 50 (data not shown) and 100 µg proteinase K ml⁻¹ (Fig. 3). However, two scFv antibodies, 3H1-D2 and E-B11, recognized antigens resistant to even high concentrations (500 µg ml⁻¹) of proteinase K (data not shown). Four scFv antibodies (E-B1, H-B6, H-B11 and 3H1-E10) recognized antigens somewhat resistant to proteinase K digestion at 100 µg ml⁻¹ (Fig. 3).

Characterization of host-cell-reactive antigens recognized by scFv antibodies

scFv antibodies 3H1-D2 and 3H1-E10 recognized a ~ 230 kDa antigen in *Chlamydia* purified from L929 cells and uninfected L929 cell lysates but this antigen was not readily detected in uninfected HeLa229 cell lysates nor in *Chlamydia* purified from HeLa229 cells (Fig. 4a), suggesting that the molecule is from the host cell. While 3H1-E10 and 3H1-D2 had similar reactivity patterns, 3H1-D2 recognized an additional ~ 42 kDa antigen (Fig. 1). The ~ 230 kDa antigen was visibly abundant in both *C. trachomatis* serovar L2 and *C. psittaci* EB lysates, yet was reproducibly less abundant in *C. trachomatis* serovar D lysates (Fig. 4a). The ~ 42 kDa antigen recognized by 3H1-D2 was evident in all strains of chlamydiae purified from both HeLa229 and L929 cells and was not observed in uninfected host cell lysates (data not shown). This suggests that the ~ 42 kDa molecule is chlamydial in origin.

By immunoblot 3H1-F2 did not reveal any antigens in purified EB but did detect a ~ 230 kDa component in L929 cells (Fig. 4b) as well as in HeLa229 cells (data not

Fig. 4. Immunoblot analysis of purified EB lysates and host-cell lysates probed with individual scFv antibodies. (a) Blot probed with scFv antibody 3H1-E10 and reacted against purified L2 EBs from L929 cells (L2/L), serovar D EBs purified from L929 cells (D/L), uninfected L929 cells (L), uninfected HeLa cells (H) and L2 EBs purified from HeLa cells (L2/H). (b) scFv antibody 3H1-F2 used to probe uninfected L929 cells (L). Calculated estimates of molecular masses (kDa) are shown on the left.

shown). Although scFv antibody 3H1-F2 detected a ~ 230 kDa antigen present in both L929 cells and HeLa229 cells as assayed by immunoblot, it exhibited a different staining morphology by immunofluorescence staining than that observed for 3H1-D2 and 3H1-E10 (Fig. 2d, e). We therefore conclude that 3H1-F2 may not bind the same antigen recognized by 3H1-D2 and 3H1-E10.

Localization and identification of a host-cell-reactive antigen recognized by scFv antibodies

scFv antibodies 3H1-E10 and 3H1-F2 were used to probe uninfected host cells to localize the recognized antigen within the host. Both fixed and unfixed live cells

Fig. 5. Immunofluorescent staining of uninfected host cells with scFv antibodies. (a, b) scFv antibody 3H1-E10 used to probe unfixed (a) and fixed (b) L929 cells. (c, d) scFv antibody 3H1-F2 used to probe unfixed (c) and fixed (d) L929 cells.

were probed. 3H1-F2 detected an antigen in fixed L929 cells but not in unfixed cells (Fig. 5c, d), suggesting that fixation was required to allow access of the scFv antibodies to an internal antigen following permeabilization of the host cell. In contrast, scFv antibody 3H1-E10, which did not recognize an antigen in HeLa229 cells, recognized an antigen in both fixed and unfixed L929 cells (Fig. 5a, b). It is likely that the antigen recognized by 3H1-E10 is surface localized, because scFv antibody binding to this antigen was not dependent on permeabilization of the cells by fixation. Similar results to those for 3H1-E10 were observed for 3H1-D2 (data not shown).

DISCUSSION

The surface molecules of infectious chlamydial EBs play essential roles in pathogenesis and virulence as they are probably represented in the initial interface for interactions with host systems, including the immune system and infection of target host cells. We exploited phage-display scFv antibody technology to dissect the complexity of chlamydial EB surface molecules. The expectation was to recover probes to a range of chlamydial antigens not limited by immunodominance; however, the quantity of the antigen present will be expected to influence selection based upon the sensitivity of the screening assay. scFv antibodies selected on the surface of purified EBs enabled the detection and identification of a variety of EB-associated antigens, some of which were chlamydial in origin whereas others were host-cell antigens. Phage-display technology allowed selection of antibodies to host molecules sequestered on *C. trachomatis* EBs without the bias of negative selection exerted by a host immune system to self molecules. Using the dot-blot assay as a marker for surface localization (Zhang *et al.*, 1987), the scFv antibodies bound *C. trachomatis* EBs, suggesting that the scFv

antibodies selected were for surface-associated components of chlamydial EBs.

Gough *et al.* (1999) attempted to select phage antibodies to *Phytophthora infestans*. It remained a question whether phage-display would be an effective tool to select a diverse population of phage antibodies to a complex antigen because they reported only selecting phage antibodies to a few different antigens despite panning and subpanning on two distinct antigen groups. Gough *et al.* (1999) speculated that recovery of so few unique phage antibodies was due to a high concentration of a single antigen. It is possible that the diversity of selected phage antibodies obtained from *Chlamydia* is due to multiple factors. Some important differences could be the variation in the starting phage-display libraries, and differences in the complexity and/or stability of the surfaces of the complex antigens.

Of the fourteen scFv antibodies that bound *Chlamydia*-associated antigens, most reacted to proteins by immunoblot. Proteins of approximately 42, 55, 69, 71, 73, 75, 110, 165 and 230 kDa were identified. By the dot-blot assay each of these proteins was found to be surface-accessible on EBs to antibody binding. Almost all the proteins were sensitive to proteinase K. Four scFv antibodies (2H-G2, H-B6, H-B11 and 2H-B9) recognized a species-specific antigen by ELISA, immunoblot (~69 kDa) and immunofluorescence. Because all four of these scFv antibodies have unique fingerprint patterns, their corresponding binding sites are different and each scFv antibody probably recognizes either a different epitope on the same antigen or, more unlikely, each scFv antibody recognizes a different antigen of similar molecular mass. scFv antibodies E-A11, E-B11 and H-E11 were genus specific by ELISA and immunoblot and recognized bands of dissimilar size in different strains of *Chlamydia*. This suggests that although epitopes are conserved across chlamydial species, there is at least size polymorphism for the analogues in *C. psittaci*. Given the high molecular mass, size polymorphism and surface localization of these antigens one could speculate that they may be members of the polymorphic membrane protein (Pmp) antigen family (Tanzer & Hatch, 2001; Tanzer *et al.*, 2001; Grimwood & Stephens, 1999; Grimwood *et al.*, 2001; Longbottom *et al.*, 1998). Likewise, scFv reactivity to a 42 kDa protein may represent recognition of a novel surface protein (protein 4) recently described by Tanzer & Hatch (2001). It was surprising that among the scFv antibodies that were reactive by immunoblot that these did not recognize the quantitatively predominant major outer-membrane protein (MOMP). This may be due to the dominance of conformation-dependent antigenicity of native MOMP (Wolf *et al.*, 2001; Fan & Stephens, 1997) and the fact that we selected antibodies with a wide spectrum of reactive intensities in the initial screening process, not merely antibodies with the strongest reactivity.

Ten scFv antibodies were reactive to a genus-specific antigen by at least one of the immunoassays. One genus-specific chlamydial antigen, previously characterized

using monoclonal antibodies, is lipopolysaccharide (LPS). LPS can typically be observed in immunoblots and in *Chlamydia*-infected cells, where immunofluorescence staining shows a markedly diffuse pattern (Stephens *et al.*, 1982). However, all but E-B11 and 3H1-D2 recognized antigens that were sensitive to proteinase K digestion, suggesting that these antibodies were not recognizing LPS but some other shared, perhaps protein molecule. Although E-B11 and 3H1-D2 recognized antigens that were resistant to proteinase K digestion their immunofluorescence staining pattern did not appear to be similar to the staining pattern observed for monoclonal antibodies to LPS, nor did the antigens resemble the immunoblot staining observed for LPS (Stephens *et al.*, 1982). Thus, phage-display technology allowed the identification of several previously undescribed surface molecules in *C. trachomatis*.

scFv antibodies 3H1-D2, 3H1-E10 and 3H1-A5, although selected from EBs and bound EBs by ELISA and dot-blot, did not react by immunofluorescence staining to chlamydial antigens within inclusions, and each reacted by immunoblot to murine L929 cell antigens in uninfected cells. Of these three scFv antibodies, only 3H1-A5 also strongly reacted to an antigen in HeLa229 cells, suggesting that its respective antigen is conserved among mammalian cell types. 3H1-D2 and 3H1-E10 both recognized a molecule of ~230 kDa detected in L929 cells but not in HeLa229 cells. Additionally, the ~230 kDa protein was apparently less abundant by immunoblots of serovar D EB lysates as compared to serovar L2 and *C. psittaci* EB lysates. Physical differences among chlamydial strains and species have been described previously (Söderlund & Kihlström, 1982); thus, it is not surprising that there are physical differences between these strains detected by scFv antibody probes.

scFv antibodies with high specificity and affinity were selected to various molecular components associated with *Chlamydia* and proved useful in a variety of analytical methods including ELISA, immunofluorescence staining and immunoblotting. While the relatively limited numbers of antibodies characterized in this study provided unexpected diversity of reactivities, many more antibodies could be selected or tested using other criteria. Unequivocal identification of protein antigens recognized by scFv antibodies requires sequence identification. Biochemical approaches such as amino-terminal microsequencing are challenging for chlamydiae because of the limited quantities of organisms that can be grown in tissue culture. However, testing scFv antibody reactivity to expressed proteins for all open reading frames identified in the chlamydial genome should permit the determination of immune-specific reactivity and the identity of the reactive antigen. This approach using phage-display antibodies has expanded the repertoire of techniques useful in the identification of antigens associated with microorganisms, especially including host antigens that would not be identified in studies based upon genome sequences or standard monoclonal antibody techniques.

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