***Chlamydia trachomatis* (L₂ Serovar) Can Be Bound, Ingested and Destroyed by Differentiated but Not by Undifferentiated Human Promyelocyte Cell Line HL-60**

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As a model system for analysing interactions between chlamydiae and myeloid cells and their precursors, we have studied binding, ingestion and destruction of *Chlamydia trachomatis* (L₂ serovar) by the human promyelocytic cell line HL-60. HL-60 cells were induced by phorbol myristate acetate (PMA) and dimethyl sulphoxide (DMSO) to differentiate along either the macrophage or the granulocyte pathway, respectively. Using an immunofluorescence assay and electron microscopy, we have shown that induced (differentiated) HL-60 cells, but not uninduced (undifferentiated) HL-60 or other cell lines treated with PMA or DMSO, exhibit increased binding, ingestion and elimination of *C. trachomatis*; these activities are associated with specific histochemical and antigenic markers of myeloid differentiation. These results suggest that myeloid cells acquire the ability to interact with and kill chlamydiae during cell development.

INTRODUCTION

Chlamydiae are obligate intracellular bacteria that are responsible for several ocular, pulmonary and genital diseases in humans (Schachter, 1978). *Chlamydia trachomatis* is the leading cause of venereal diseases in humans, surpassing both *Neisseria gonorrhoeae* and herpes simplex in frequency (Thompson & Washington, 1983), and endometritis and salpingitis caused by chlamydiae are responsible for a large percentage of cases of female infertility (Cleary & Jones, 1985). Trachoma is a major, world-wide ocular infection, causing over two million cases of preventable blindness each year (Dawson, 1982). Chlamydiae are also the primary agents of infantile pneumonia and conjunctivitis (Bell, 1985) and *C. psittaci* may be an important cause of adult pneumonia syndromes (Grayston *et al.*, 1986).

Chlamydiae exhibit a unique and complex life cycle (Moulder *et al.*, 1984) which includes a metabolically inactive but infectious form called an elementary body (EB) and a larger, metabolically active, replicating form called a reticulate body (RB). *C. trachomatis* binds to epithelial cells, fibroblasts (Storz & Spears, 1977; Croy *et al.*, 1975), mouse peritoneal macrophages (Kuo, 1978, 1979), human peripheral blood monocytes, granulocytes and B cells (Bard & Levitt, 1986), and several human lymphoblastoid cell lines (Bard & Levitt, 1985). Although *C. trachomatis* binds to various cell types, production of infectious organisms, which involves successful binding, ingestion, replication and release of bacteria, occurs primarily in fibroblasts and epithelial cell lines (Storz & Spears, 1977; Croy *et al.*, 1975).

The role of phagocytic cells during natural infection is to ingest and eliminate micro-organisms, but the mechanisms which inhibit chlamydial growth within these cells are poorly

Abbreviations: DMSO, dimethylsulphoxide; EB, elementary body; ID₅₀, 50% infectious dose for McCoy cells; i.f.u., inclusion-forming unit; LGV, lymphogranuloma venereum; PMA, phorbol myristate acetate; PMN, polymorphonuclear leucocyte; RB, reticulate body.

understood. Investigations of the interactions between chlamydiae and human peripheral blood monocyte/macrophages and polymorphonuclear leucocytes (PMNs) have been limited due to the low number of monocytes in peripheral blood and the short life span of granulocytes in tissue culture. Also, the ability of myeloid cells to bind, ingest and destroy chlamydiae during cell differentiation has been difficult to examine.

We have previously reported binding of *C. trachomatis* (L₂ serovar) to a subpopulation (20–30%) of human peripheral blood monocytes and to approximately 60–80% of human peripheral blood granulocytes (Bard & Levitt, 1986). Furthermore, we have demonstrated that human peripheral blood monocytes do not permit chlamydial growth and that granulocytes destroy these bacteria after ingestion (Zvillich & Sarov, 1985; Yong *et al.*, 1986). To define the ability of myeloid cells at different stages of development to interact with chlamydiae, we have analysed binding, ingestion and inhibition of growth of *C. trachomatis* (L₂/434/Bu, LGV biovar) in human monocyte-like and granulocyte-like cells chemically induced from the same promyelocytic cell line, HL-60 (Rovera *et al.*, 1979; Collins *et al.*, 1978). Our data indicate differentiation-associated specific binding of chlamydiae to induced HL-60 cells, consistent with the acquisition of specific binding molecule(s) for chlamydiae during myeloid differentiation. Binding is followed by ingestion and chlamydial growth retardation or destruction.

METHODS

Bacteria. *Chlamydia trachomatis* (L₂/434/Bu, LGV biovar), isolated from infected McCoy cells and purified as previously described (Bard & Levitt, 1984), was used. The infectious titre of bacteria, represented either as i.f.u. per ml inoculum (supernatant) or as 50% infectious dose (ID₅₀), was determined by titration on McCoy cells (Bard & Levitt, 1984; Rothmel *et al.*, 1983a). Total numbers of bacterial particles were determined with the aid of a micrometer by a modification of the method described by Reeve & Taverne (1962), utilizing fluorescent antibody directed against chlamydiae (see below) to detect single chlamydial particles.

Growth and induction of the HL-60 cell line. The human promyelocyte cell line HL-60 was obtained from the American Type Culture Collection and grown in RPMI 1640 medium (Gibco) with 2 mM-glutamine, 10 µg gentamicin ml⁻¹ and 20% (v/v) foetal bovine serum (complete medium). Cells were tested weekly for mycoplasma contamination (Chen, 1977). Cells used were taken from exponentially growing cultures.

On day 0, 1.25% (v/v) dimethyl sulphoxide (DMSO) (Sigma) or 16 nM-phorbol myristate acetate (PMA) (CCR, Inc.) were added to complete medium, and DMSO- or PMA-induced cells were incubated for 7 d and 2–4 d, respectively. Mean cell viability for uninduced, DMSO-induced and PMA-induced HL-60 cells was 95%, 85% and 80%, respectively, as determined by trypan blue dye exclusion. Uninduced and induced cells were harvested and washed three times prior to the binding assay (see below). Adherent PMA-treated cells were removed with a rubber policeman (nonadherent cells were discarded).

Binding assay. The number of bacteria binding to cells was determined as previously described (Bard & Levitt, 1986). Briefly, 10⁶ HL-60 cells in 0.1 ml complete medium were added to autoclaved screw-cap 15 × 45 mm vials (Thomas Scientific), and 0.1 ml purified *C. trachomatis* [multiplicity of infection (m.o.i.) ratio (EB: host cell) 30–50:1] was incubated with cells without centrifugation at 37 °C for 1 h. Cells were then sedimented through foetal bovine serum at 75 g for 5 min to separate cell-bound bacteria from unbound bacteria. The cell pellet was resuspended in phosphate-buffered saline (0.15 M-NaCl in 0.1 M-sodium phosphate, pH 7.2) containing 0.02% NaN₃ (PBS-azide) and 0.5% bovine serum albumin (BSA), washed twice, then cytocentrifuged onto glass microscope slides. The slides were fixed in either 100% methanol or acetone for 5 min and stained in moist chambers for 30 min with either fluorescein-conjugated mouse monoclonal antibody specific for L₂ serotype (kindly provided by Dr Milton Tam, Genetic Systems Corp., Seattle, WA, USA) (Stephens *et al.*, 1982) or an irrelevant fluorescein-conjugated mouse IgG myeloma, MOPC-21 (generously provided by Dr J. Kearney, University of Alabama, Birmingham, AL, USA). Other negative controls which provided evidence for staining specificity included the use of protease-treated chlamydiae, mock-infected McCoy cell inocula and anti-L₂ antibody reacted with host cells without prior bacterial incubation (Bard & Levitt, 1985; Levitt *et al.*, 1986). The slides were washed, mounted and examined for fluorescence using a Nikon Optiphot fluorescent microscope; photomicrographs were obtained with a UFXII automatic photographic system and Kodak daylight 400 slide film. Only cells which bound three or more bacteria particles were scored as positive for chlamydiae; more than 1000 cells and at least 10 fields were analysed for each experiment.

Surface-associated chlamydiae were unequivocally demonstrated by observing: (1) comparable percentages of positive cells stained before and after fixation (detecting only surface-bound bacteria and both surface-bound and intracellular bacteria, respectively); (2) diminished immunofluorescent staining after removing externally bound bacteria by protease treatment (Byrne, 1978) compared to untreated preparations. In both cases, either McCoy cells (as a control) or induced HL-60 cells were incubated for 20 min with chlamydiae. Cells were then prepared for

staining as described above in the first case, or washed and protease-treated to remove surface-bound bacteria as described below (*Ingestion of bacteria*) for the second case. Protease-treated chlamydiae are not reactive with the anti-L₂ antibody used in this study (unpublished observations and Stephens *et al.*, 1982). The specificity of this technique for demonstrating chlamydial binding as opposed to ingestion has been confirmed using several other leucocyte cell lines (Bard & Levitt, 1985) and by flow cytometry (Levitt *et al.*, 1986).

In some experiments, 10⁶ uninduced or DMSO-induced HL-60 cells were incubated with mouse monoclonal antibody against the myeloid cell marker MY4 (Coulter Immunology) followed by rhodamine-conjugated goat anti-mouse Ig (Griffin *et al.*, 1981), before doing the chlamydial binding assay as described above. No staining was observed when cells were incubated with the latter reagent alone. Indirect immunostaining with anti-MY4 antibody prior to chlamydial incubation had no effect on the percentage of cells positive for chlamydiae.

Histological and cytochemical characterization of cells. Acquisition of α -naphthyl acetate esterase was analysed by non-specific esterase staining as described in Sigma Technical Bulletin no. 90. In some experiments, the percentage of chlamydial-binding, esterase-positive cells was assessed in cell preparations with bound chlamydiae by histochemical staining for non-specific esterase followed by fluorescent staining for bound L₂ serovar as described above. Morphology of cells was analysed by Wright-Giemsa staining. The ability of uninduced and induced HL-60 cells to synthesize proteins and proliferate was determined by measuring incorporation of [³H]leucine [1 μ Ci ml⁻¹; sp. act. 50 Ci mmol⁻¹ (1850 GBq mmol⁻¹); New England Nuclear] and [³H]thymidine [5 μ Ci ml⁻¹; sp. act. 6.7 Ci mmol⁻¹ (248 GBq mmol⁻¹); New England Nuclear] into methanol-insoluble fractions (Bard & Levitt, 1984).

Histological, cytochemical and morphological criteria, as previously described in detail (Rovera *et al.*, 1979), used for defining changes in promyelocyte HL-60 cells treated with either DMSO or PMA were as follows. Promyelocytes (uninduced HL-60 cells) possessed large, round nuclei containing two to four nucleoli and dispersed chromatin, basophilic cytoplasm with granules, high nuclear/cytoplasmic ratio, and the ability to proliferate and synthesize proteins but not to phagocytose (tested with *Staphylococcus aureus*); they did not exhibit non-specific esterase staining. More mature myeloid cells (DMSO-treated HL-60 cells) were smaller, with a diminished nuclear/cytoplasmic ratio, few nucleoli or cytoplasmic granules, marked segmentation and indentation of nuclei, a high level of protein synthesis but no DNA synthesis, phagocytic ability and negative non-specific esterase staining. Cells of macrophage lineage (PMA-treated HL-60 cells) had no cytoplasmic granules, several nucleoli, large droplets and vacuoles, irregularly-shaped nuclei and long, thin cytoplasmic processes, and they exhibited a high level protein synthesis but no DNA synthesis; they possessed phagocytic ability, adhered to plastic and showed 95% non-specific esterase-positive staining.

Ingestion of bacteria. *C. trachomatis* or *Staphylococcus aureus* Cowan I (Pansorbin; Calbiochem) at a bacteria:cell ratio of 10:1 were added to 5 $\times$ 10⁶ PMA-treated, DMSO-treated or uninduced cells. Cells were incubated in complete medium at 37 °C for 4 h, washed in Hanks' balanced salts solution (HBSS) (no serum), and surface-bound bacteria were removed by treatment in suspension with 4 mg pronase ml⁻¹ (pronase E, type XIV, from *Streptomyces griseus*; Sigma) (Byrne, 1978); control cells were mock-treated with HBSS alone for 30 min at 37 °C. Cells were sedimented through foetal bovine serum gradients, washed three times at 75 g, and cytocentrifuged onto slides. Intracellular bacteria were determined either by fluorescence staining, as described above (*Binding Assay*), for chlamydiae, or by phase-contrast microscopy for *S. aureus*.

Growth of C. trachomatis. Purified *C. trachomatis* in 0.1 ml (m.o.i. = 10:1, able to infect >90% of the susceptible McCoy cells) were added to 1 $\times$ 10⁶ cells for 1 h at 37 °C, then the cells were washed twice with culture medium. After 24–32 h, the percentage of cells which supported replication of bacteria was determined by immunofluorescent staining of inclusion-containing cells (indicative of bacterial proliferation), as described above (*Binding assay*). For infectivity (yield of bacteria), cells were harvested after exposure to bacteria, washed, resuspended in 2.0 ml complete medium and incubated at 37 °C for 48–72 h in 60 mm tissue culture dishes (5% CO₂:95% air). Cells were then harvested, sonicated for 45 s (setting of 30, Fisher dismembrator, model 30) and released bacteria were quantified using McCoy cells (Bard & Levitt, 1984; Rothermel *et al.*, 1983a).

Transmission electron microscopy. Samples were fixed with 4% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4), washed and postfixed in 1% (w/v) osmium tetroxide in 0.1 M-cacodylate buffer (pH 7.4), for 1 h. The fixed cells were placed in 0.5% aqueous uranyl acetate for 1 h, then dehydrated, embedded and sectioned with a glass knife. Thick sections were stained with toluidine blue. Thin sections were post-stained with 2% (w/v) aqueous uranyl acetate followed by Reynold's lead citrate and examined with a Zeiss EM9S-2 electron microscope.

Statistical analysis. Statistical analysis was done by Student's *t*-test.

RESULTS

Binding of C. trachomatis by uninduced, PMA-induced and DMSO-induced HL-60 cells

The majority of HL-60 cells were promyelocytic according to morphological and histochemical criteria; however, ~5% of cells in culture spontaneously differentiated and

showed phenotypic characteristics of more mature myeloid cells (myelocytes, metamyelocytes, band forms and a few segmented or polymorphonuclear forms: Rovera *et al.*, 1979; Collins *et al.*, 1978) (data not shown). We evaluated the number of cells binding chlamydiae by staining the cell-associated bacteria with mouse monoclonal antibody specific for *C. trachomatis*, L₂ serotype (Stephens *et al.*, 1982), using a direct immunofluorescence assay. Minimal binding of chlamydiae to uninduced cells was observed (~4% of the cells positive for chlamydial binding) (Table 1, Fig. 1*d, f*). In addition, a minor subpopulation of uninduced HL-60 cells (~3%) expressed the myeloid differentiation antigen MY4 (Griffin *et al.*, 1981) (Table 1, Fig. 1*d, e*). The minor subpopulation to which chlamydiae attached (~4%) corresponded to the cells which also displayed the MY4 marker (Fig. 1*d-f*), suggesting that the chlamydiae bound preferentially to myeloid cells which had progressed beyond the promyelocytic stage. This interpretation was confirmed by incubating HL-60 cells with 1.25% DMSO for 7 d to induce differentiation along the granulocytic path. Almost 80% of these treated cells phagocytosed formalin-fixed *S. aureus*, compared with fewer than 1% of untreated HL-60 cells, indicating their differentiation into professional phagocytes. Large increases in the percentage of MY4-positive cells (15-fold) (Fig. 1*a, b*) and chlamydia-binding cells (7-fold) (Fig. 1*a, c*; Fig. 2*a, b*) were observed in these maturing myeloid (granulocytic) cells when compared with uninduced, undifferentiated promyelocyte cells ($P < 0.01$ for both parameters; Table 1, Fig. 1*d-f*). The difference in the percentage of cells binding chlamydiae and those expressing the MY4 marker (32% vs 53%) could not be accounted for by the interference of bacterial binding by the anti-MY4 antibodies. Similar percentages of chlamydia-positive cells were obtained when binding was performed either before or after MY4 staining (see Methods). These data suggested either that not all 'differentiated' HL-60 cells bound *C. trachomatis* L₂ or that the sensitivity of detecting chlamydial binding was less than that of detecting MY4. However, the majority of chlamydia-binding cells were MY4 positive (86%; Table 1, Fig. 1*a-c*).

More than 90% of HL-60 cells exposed to PMA for 4 d were adherent and resembled cells of the monocyte/macrophage lineage. Of the adherent cells, 95% stained for non-specific esterase and 90% ingested formalin-fixed *S. aureus*. Approximately 50% of PMA-induced cells bound chlamydiae (Table 1) ($P < 0.01$ compared to uninduced cells). In addition, the ability of PMA-induced cells, representing cells of the monocyte/macrophage lineage, to bind chlamydiae was coincident with the expression of non-specific esterase, a marker for promyelocyte to macrophage differentiation/maturation (Fig. 3*a, b*). These data demonstrate that differentiation of promyelocyte cells along both myeloid and monocyte/macrophage lineages results in the coincidental acquisition of myeloid markers (MY4 and non-specific esterase, respectively) and ability to bind *C. trachomatis*.

Minimal growth of C. trachomatis in HL-60 cells

C. trachomatis entered and replicated within only a minor subpopulation of uninduced HL-60 cells (~1%, determined by assaying the percentage of cells containing inclusions 24–32 h post-inoculation; Table 1). The yield of bacteria (approx. 9.8×10^5 i.f.u. ml⁻¹) was less than 0.1% of yields obtained from a comparable number of McCoy cells (data not shown). To further demonstrate that the majority of HL-60 cells were unable to support growth of chlamydiae (and that not merely a random fraction of cells became infected), we added increasing numbers of bacteria to uninduced cells (2–50 ID₅₀ for McCoy cells); if infection were dose related, then increasing the number of chlamydiae should elevate the percentage of infected HL-60 cells. An identical titration was performed on the reference cell line, McCoy cells. At all input bacterial doses which permitted 90% or greater binding to and infectivity of McCoy cells (10–50 ID₅₀), 5% or fewer HL-60 cells demonstrated bacterial binding or inclusions (Fig. 4). Therefore, only a minor subpopulation (~1%) of HL-60 cells possessed receptors for *C. trachomatis* and permitted its growth.

The number of cells binding chlamydiae increased more than 12-fold and 7-fold after PMA- and DMSO-induction, respectively; however, the yields of bacteria and the percentages of cells supporting growth of chlamydiae were comparable to those for the uninduced cell population (Table 1; $P > 0.05$). The percentage of cells supporting growth of chlamydiae at about 48 h post-

Fig. 1. Differentiation-associated specific binding of *C. trachomatis* (LGV biotype) by uninduced and DMSO-induced HL-60 cells. DMSO-induced (*a-c*) and uninduced (*d-f*) HL-60 cells were reacted with mouse monoclonal antibody against the myeloid cell marker MY4 followed by rhodamine-conjugated goat anti-mouse Ig, then incubated with chlamydiae, fixed and stained with fluorescein-conjugated mouse monoclonal antibody specific for L₂ serotype, as described in Methods. (*a, d*) Phase contrast; (*b, e*) same respective field showing MY4-positive cells (rhodamine-stained); (*c, f*) same respective field showing surface-bound chlamydiae (fluorescein-stained). No fluorescein staining was observed without incubation of cells and bacteria. The diffuse staining of chlamydial particles was due to visualization through several planes of focus. (*a-c*) and (*d-f*) represent two separate fields. Separate filters were utilized for independent viewing of rhodamine and fluorescein staining. Bars 20 μ m (magnification the same in each photograph).

Table 1. *Binding and growth of C. trachomatis in uninduced and induced HL-60 cells*

HL-60 cells were cultured without inducer, or in the presence of PMA for 2-4 d or DMSO for 4 d, as described in Methods. The results are means $\pm$ SEM from 3-12 experiments.

Inducer	Percentage of:				Yield of <i>C. trachomatis</i> ($10^{-5} \times$ i.f.u. ml $^{-1}$) $\dagger$
	Chlamydia-binding cells*	MY4 ⁺ cells*	Chlamydia-binding cells that were MY4 ⁺⁺	Inclusion-containing cells $\dagger$	
None	4.4 $\pm$ 0.9	3.3 $\pm$ 0.3	85.5 $\pm$ 1.2	1.3 $\pm$ 0.3	9.8 $\pm$ 3.5
PMA	53.7 $\pm$ 1.9§	ND	ND	1.5 $\pm$ 0.8	6.5 $\pm$ 5.8
DMSO	31.8 $\pm$ 3.5§	53.3 $\pm$ 9.4§	86.8 $\pm$ 5.7	2.4 $\pm$ 1.1	1.9 $\pm$ 1.3

* Chlamydia-binding cells were enumerated using fluorescein-conjugated mouse monoclonal antibody specific for the L₂ serotype of *C. trachomatis*. MY4-positive cells were assessed by viable staining with mouse monoclonal antibody against the myeloid marker MY4 followed by rhodamine-conjugated goat anti-mouse Ig. No staining was observed when cells were incubated with the latter reagent alone. The percentage of chlamydia-binding cells that were MY4 positive was enumerated by assessing the percentage of L₂-positive cells that co-stained with MY4 antibody. More than 10^3 cells were counted for each experiment. ND, Not done.

$\dagger$ Cells incubated with *C. trachomatis* were harvested 24-32 h after culture, fixed, stained with fluorescein-conjugated monoclonal anti-L₂ and examined for the percentage of inclusion-containing cells. More than 10^3 cells were counted for each experiment.

$\ddagger$ Cells were cultured for 48 h after exposure to bacteria then harvested and sonicated; the released bacteria were quantified using McCoy cells. Comparable numbers of cells were present in sonicated suspensions.

§ $P < 0.01$ in comparison to uninduced HL-60 cells.

Fig. 2. Binding and ingestion of *C. trachomatis* by DMSO-induced HL-60 cells. Chlamydiae were centrifuged for 45 min onto day 7 DMSO-induced HL-60 cells and stained with mouse monoclonal antibody specific for *C. trachomatis* (L₂ serotype) (*a*, *b*). In some experiments (*c*, *d*) cells were incubated for 4–5 h after centrifugation of bacteria to permit ingestion of surface-bound bacteria (see Table 2). Uningested bacteria were then removed by protease treatment and cells were stained as above. (*a*) Phase-contrast micrograph and (*b*) fluorescent micrograph of surface-bound chlamydiae. The arrow indicates a cell with bound bacteria. Chlamydiae are clustered on the surface of cells, in many instances. Some bacteria which are in a different plane of view are out of focus. (*c*) Phase-contrast micrograph and (*d*) fluorescent micrograph of ingested chlamydiae. The arrow indicates a DMSO-induced cell that has ingested bacteria. Bar, 20 μ m (magnification the same in each photograph).

inoculation was 1–2% of total cells (uninduced or induced) and the yield of infectious progeny at 72 h post-inoculation was $1-4 \times 10^5$ i.f.u. ml⁻¹ for three separate experiments. These data were not significantly different from observations at earlier time points (see Table 1). Therefore, bacteria binding to the majority of induced cells were either not being ingested or were phagocytosed and their growth was inhibited. Both possibilities were evaluated using immunofluorescence and electron microscopy.

Fig. 3. Binding of *C. trachomatis* by non-specific-esterase-positive PMA-induced HL-60 cells. PMA-induced HL-60 cells were incubated with *C. trachomatis*, sedimented through foetal bovine serum and cytocentrifuged onto glass slides. Slides were fixed, stained for non-specific esterase and then incubated with fluorescein-conjugated mouse monoclonal antibody against chlamydiae, as described in Methods. (a) Non-specific-esterase-positive, PMA-induced HL-60 cells. All cells in the field are esterase-positive, as indicated by the presence of black granules in the cytoplasm. (b) Fluorescein-positive, surface-bound chlamydiae on PMA-induced HL-60 cells. Some cells which are esterase-positive do not bind chlamydiae. Fixation for esterase staining results in a significant degree of fluorescence quenching. Bars, 10 μ m.

Fig. 4. Minimal growth of *C. trachomatis* in uninduced HL-60 cells. Increasing numbers of infectious bacteria (2–50 ID₅₀) were centrifuged onto uninduced HL-60 cells (●) or McCoy cells (▲). The percentage of inclusion-containing cells 24–32 h post-inoculation was scored by fluorescence microscopy. Data are representative of three separate experiments.

Ingestion of *C. trachomatis*

Phagocytosis of *C. trachomatis* by HL-60 cells was assessed by immunofluorescence after protease treatment to remove bound but not ingested bacteria (Byrne, 1978). Fewer than 10% of PMA-induced HL-60 cells incubated with bacteria for 20 min were stained after protease treatment, compared to approximately 30% in non-protease-treated controls (data not shown). However, when PMA-induced HL-60 cells were incubated for 4 h with *C. trachomatis*, no reduction occurred in the percentage of cells stained for chlamydiae after protease treatment (Table 2). Thus, chlamydiae appeared to be ingested by PMA-induced cells by 4 h post-inoculation. Similarly, chlamydiae were avidly ingested by DMSO-induced HL-60 cells (Table 2, Fig. 2). Not all PMA-induced cells that bound chlamydiae were able to ingest the bacteria (Tables 1 and 2). Neither DMSO nor PMA caused enhanced binding or ingestion of *C. trachomatis* by lymphoblastoid cell lines Daudi, F4 and LR-2, or the histiocytic cell line U937, under identical experimental conditions as described for HL-60 cells (unpublished observations). In addition, only 2–4% of day 1 DMSO-treated or 2 h PMA-treated HL-60 cells could bind chlamydiae; similarly, fewer than 1% of these cells contained inclusions. These results

Fig. 5. Ultrastructural evaluation of *C. trachomatis* in PMA-induced HL-60 and McCoy cells. (a) McCoy cell 27 h post-inoculation. Note the large inclusion containing many bacteria at various stages of development (i.e. larger RBs, small and condensed EBs and intermediate-sized transitional forms). (b) PMA-induced HL-60 cell 27h post-inoculation. A representative inclusion-containing cell is shown to permit comparison with the McCoy cell inclusions in (a). The inclusions are small and contain few bacteria, most of which are RB forms. (c) PMA-induced HL-60 cell 3 h post-inoculation. (d) PMA-induced HL-60 cell 19 h post-inoculation. In (c) and (d), the arrowhead designates one of several degraded bacteria and the arrow indicates an intact bacterium enclosed in a phagosome. Bars, 1 μ m.

suggested that true changes in the phenotype/function/maturation of HL-60 cells, and not merely exposure to PMA and DMSO, caused enhanced binding, ingestion and elimination of *C. trachomatis* by the treated cell line (see below).

Electron microscopic analyses of C. trachomatis-infected HL-60 cells

Since chlamydiae were being ingested by significantly greater numbers of HL-60 cells (approx. 25-fold) following treatment with PMA (Table 2), yet no increase in the percentage of

Table 2. *Ingestion of C. trachomatis by uninduced and induced HL-60 cells*

C. trachomatis (m.o.i. 10:1) were centrifuged onto 5×10^6 PMA-treated, DMSO-treated and uninduced cells. Cells were incubated at 37 °C for 4–5 h, harvested, washed in Hanks' balanced salts solution (HBSS) (no serum), and surface-bound chlamydiae were removed by treatment with 4.0 mg pronase ml⁻¹ in HBSS or mock-treated with HBSS alone for 30 min at 37 °C. Cells were sedimented through foetal bovine serum gradients, cytocentrifuged onto slides and stained as described in Methods. More than 1000 cells and more than 10 fields were examined. Data from two representative experiments (from a total of four experiments) are shown.

Inducer	Percentage of cells ingesting chlamydiae			
	Expt 1		Expt 2	
	– Pronase	+ Pronase	– Pronase	+ Pronase
None	1	0	3	1
PMA	25	14	31	31
DMSO	30	26	26	28

Table 3. *Quantity and developmental forms of chlamydiae inside inclusions of McCoy cells and PMA-induced HL-60 cells*

McCoy cells and PMA-induced HL-60 cells were incubated with *C. trachomatis* for 27 h as described in Methods. The cells were harvested and processed for transmission electron microscopy. The types of bacteria (i.e. EBs, transitional forms or RBs) found in 10 different inclusions of 10 representative cells from three different thin sections cut from infected McCoy and PMA-induced HL-60 cells were analysed.

Cell type	Total no. of different chlamydial forms in three representative sections of inclusions			Mean no. of chlamydiae per section of inclusion*
	EB	Transitional	RB	
McCoy	144	164	104	137 ± 16
PMA-induced HL-60	0	2	31	10 ± 1.9†

* The number of bacterial particles in 10 sections of inclusions from 10 different cells was scored and the mean number of chlamydiae per section ± SEM was determined.

† $P < 0.01$ comparing McCoy and PMA-induced HL-60 cells.

cells supporting growth or in the numbers of released infectious bacteria was found (Table 1), growth of chlamydiae within the induced cells was presumably inhibited. This conclusion was supported by electron microscopic examination of induced HL-60 cells after chlamydial infection.

Toluidine-blue-stained sections of PMA-induced HL-60 cells 27 h post-inoculation revealed that fewer than 5% of the cells contained inclusions, thus confirming the immunofluorescence data. In contrast, 80–90% of McCoy cells at this time supported growth of chlamydiae. Electron microscopic examination of inclusions in PMA-induced HL-60 cells revealed dramatic differences in the quantity and types of bacteria compared to inclusions found in McCoy cells (Table 3). The inclusions in induced HL-60 cells were smaller and contained 10-fold fewer bacteria which were less mature (95% of the bacterial particles were RBs) compared with McCoy cells (EBs, transitional forms and RBs; Fig. 5*a, b*). Thus, PMA-induced HL-60 cells, which resemble cells of the monocyte/macrophage lineage, can both inhibit and retard growth of chlamydiae.

The PMA-induced HL-60 cells were also examined after 3 and 19 h incubation with *C. trachomatis*. Chlamydiae were identified as single particles inside cytoplasmic vesicles as well as on the cell surface (Fig. 5*c, d*). Some bacteria appeared partially degraded, while others were intact. Examination of induced HL-60 cells at 48–60 h post infection revealed similar results (48–60 h infected McCoy cells were extremely fragile and could not be evaluated).

DISCUSSION

Human and animal chlamydial infections are associated with polymorphonuclear and mononuclear (monocyte/macrophage) leucocyte infiltration of affected tissues (Lammont & Nichols, 1981); therefore, the roles of these leucocyte populations in protecting and resolving chlamydial infections in the host may be important (Benedict & McFarland, 1958; Brunham *et al.*, 1985; Rank *et al.*, 1985). Several processes seem to occur when chlamydiae of the LGV biovar confront induced HL-60 cells, which morphologically and functionally resemble phagocytes: a proportion of induced HL-60 cells will bind chlamydiae and a subpopulation of cells which bind chlamydiae will also ingest them. Once inside the cells, the bacteria either (i) are destroyed, (ii) persist as single bacteria, or (iii) initiate a severely retarded growth (replication) cycle. These latter bacteria may eventually replicate or persist inside the cells, possibly contributing to latent chlamydial infections (Rothermel *et al.*, 1983*a, b*; Byrne & Krueger, 1983). This scheme of events probably reflects the complexity of the various defence mechanisms available to both host and bacteria during an infection.

Differentiation of the promyelocyte cell line HL-60 to phagocytes results in coincidental expression of myeloid maturation markers (Griffin *et al.*, 1981) and the ability to bind chlamydiae. This specific acquisition of bacterial binding suggests that appearance of binding molecule(s) for chlamydiae and specialized myeloid markers (MY4, non-specific esterase) is the result of expression of differentiation-specific antigens in maturing phagocytes. Since there is also increased association of other bacteria – *S. aureus* (see text) and *Escherichia coli* (data not shown) – with chemically induced (differentiated) HL-60 cells, this differentiation-linked bacterial association is probably not specific for chlamydiae but instead represents a generalized mechanism that occurs during phagocyte maturation. Such recognition of pathogens by mature, and not immature, phagocytes might play a major role in non-specific host defences against microbial invasion.

Studies of the interactions between chlamydiae and phagocytic cells of human origin have been limited. The mechanisms by which such cells protect against chlamydial infections are unclear. Our findings are in agreement with those of Zvillich & Sarov (1985) and Yong *et al.* (1986), who described two types of EB-containing phagosomes in chlamydia-infected human PMNs: one appeared to contain a single intact EB while the other was larger, containing intact or degenerated EBs, or both. Yong *et al.* (1982) also demonstrated complete inactivation of *C. trachomatis* (L₂ serovar) as early as 30 min after incubation with human PMNs. Although respiratory burst is an important mechanism for microbial killing by macrophages and granulocytes (Gabig & Baboir, 1981), the bacteriocidal activity for *C. trachomatis* of PMNs from individuals with chronic granulomatous disease (CGD) and normal individuals was comparable, suggesting that oxygen-dependent antimicrobial systems were not essential for killing chlamydia (Yong *et al.*, 1982). Oxidatively deficient monocyte-derived macrophages from patients with CGD can display effective bacteriostatic activity against *C. psittaci* when stimulated with mitogen-induced lymphokines (Murray *et al.*, 1983). γ -Interferon present in concanavalin-A-induced lymphokines can activate human monocyte-derived macrophages to cause this inhibitory effect (Rothermel *et al.*, 1983*b*). Although uninduced HL-60 cells produce α and β interferon (Matsuyama *et al.*, 1982), it is not known whether PMA-induced HL-60 cells synthesize interferon. If they do, interferon may be responsible for the reduction in the growth of chlamydiae in these cells which could lead to latent infections (Rothermel *et al.*, 1983*b*; DeLaMaza *et al.*, 1984).

In this study, we have analysed binding and infectivity of *C. trachomatis* in the human promyelocytic cell line HL-60 before and after induction with the tumour promotor PMA and the nonpolar solvent DMSO. PMA causes differentiation/maturation along monocyte/macrophage lineages, whereas DMSO promotes granulocyte development (Rovera *et al.*, 1979; Collins *et al.*, 1978). Induction of HL-60 cells is not only associated with the development of certain enzymes (Newburger *et al.*, 1979) and surface molecules (Griffin *et al.*, 1981; Rovera *et al.*, 1982; May *et al.*, 1984) but also, as we have demonstrated, with changes in the ability of these cells to bind, ingest and inhibit growth of chlamydiae. Acquisition of a specific receptor for chlamydiae

during differentiation along the myeloid pathway is a likely possibility. Our results further suggest that the ability of myeloid cells to interact with and destroy chlamydiae correlates with distinct stages of differentiation; immature cells may be unable to bind, ingest and destroy bacteria. This finding may be particularly significant for individuals in whom myeloid cell development is hindered, e.g. during immunosuppressive therapy.

We have recently described chlamydial binding to 70% of normal human peripheral blood monocytes using a binding protocol similar to that described here (Bard & Levitt, 1986). The availability of chemically inducible myeloid-cell-line analogues of mature phagocytes, possessing a subpopulation of cells (~50%) which bind chlamydiae, should facilitate characterization of a 'putative' binding molecule or receptor for chlamydiae and potentially other micro-organisms. Such systems will be useful for analysing the molecular mechanisms involved in binding and growth inhibition of chlamydiae by human monocytes and PMNs and host-microbe interactions during phagocytic cell differentiation.

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