Complement Activation and Stimulation of Chemotaxis by
Chlamydia trachomatis

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The stimulus for the migration of polymorphonuclear leukocytes (PMNs) in acute chlamydial infection was studied in vitro by examining the chemotaxigenic effect of L2 and DE Chlamydia trachomatis elementary bodies (EB) upon the plasma of three healthy donors. In each individual experiment, chemotactic response was assessed with PMNs and plasma from the same respective donor, and no specific antibodies against C. trachomatis were detected in the plasma of any donor. Chemotaxis was observed in an agarose plate assay and was quantitated as the chemotactic differential, or CD (directed migration of PMNs minus random movement of PMNs). For each donor, the mean CD was significantly greater (P < 0.005) when plasma preincubated for 2 h with L2 EB was used as the chemoattractant than when (i) plasma alone, (ii) plasma preheated to 56°C for 30 min before incubation with L2 EB, or (iii) L2 EB in phosphate-buffered saline (PBS) was used as the potential chemoattractant. Similarly, in the one donor in whom DE EB were studied, the mean CD was also significantly greater (P < 0.005) for plasma preincubated with DE EB as compared with (i) plasma alone or (ii) DE EB in PBS. Complement activation by C. trachomatis EB was assessed by radioimmunoassay for C5a des-arginine in all chemoattractant preparations used in the chemotaxis assay. Mean C5a des-arginine levels were high in plasma samples preincubated with L2 EB (171.00 ± 10.64, 107.00 ± 4.76, and 89.70 ± 1.74 ng per ml) or DE EB (37.40 ± 15.76 ng per ml) but were undetectable (<4.00 ng per ml) in (i) plasma alone, (ii) preheated plasma incubated with L2 EB, and (iii) PBS containing L2 EB. Thus, L2 EB and DE EB of C. trachomatis exert a chemotaxigenic effect upon normal antibody-negative plasma, and this effect is at least in part a result of complement activation and generation of the potent chemotaxin C5a.

In the past decade, Chlamydia trachomatis has been identified as a significant and frequent human pathogen. C. trachomatis is now recognized to be a major cause of sexually transmitted genitourinary disease and has been associated with urethritis, epididymitis, and proctitis in the male and “acute urethral syndrome,” cervicitis, salpingitis, and perihepatitis in the female (2). It is also an important pathogen in neonates and may result in conjunctivitis or pneumonia in this setting (10). In addition, certain serovars are responsible for specific disease entities, such as trachoma and lymphogranuloma venereum (2).

Although C. trachomatis is an obligate intracellular bacterium, there is an intense inflammatory response to infection with this organism. In the initial phase, the cellular component of this response consists primarily of polymorphonuclear leukocytes (PMNs) and macrophages (13, 14). Yong et al. (24) demonstrated in vitro that PMNs were capable of phagocytosing and killing elementary bodies (EB) of C. trachomatis, but little else is known about the interaction of this organism with PMNs. In addition, the interaction between C. trachomatis and other components of the acute inflammatory response, such as the complement system, have received limited study.

The objective of this study was to examine in vitro a possible stimulus for the migration of PMNs seen in acute chlamydial infection. We report here that C. trachomatis EB are capable of activating the complement cascade and in doing so exert a potent chemotaxigenic effect upon normal plasma.

MATERIALS AND METHODS

Tissue culture cells. McCoy cells (Flow Laboratories, Inc., McLean, Va.) were maintained in 75-cm² Falcon plastic tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif.) and incubated at 37°C.

Media. Cells were grown in minimal essential medium with Earle salts (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine (Eastman Kodak Co., Rochester, N.Y.), 10% heat-inactivated fetal bovine serum (GIBCO), 8 μg of gentamicin (Schering Canada, Pointe Claire, Canada) per ml, 25 μl of nystatin (E. R. Squibb & Sons, Montreal, Canada) per ml, and 7 mM sodium bicarbonate to pH 7.5. This medium was called chlamydia medium with antibiotics. For inoculation of stock chlamydial cultures, chlamydia medium with antibiotics was supplemented with glucose (to a final concentration of 0.5%), 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.), and 1 μg of cycloheximide (BDH Chemicals, Toronto, Canada) per ml. This medium was called CMGA-HEPES with cycloheximide.

C. trachomatis. Two human genital serovars of C. trachomatis, a lymphogranuloma venereum L2/434 serovar and a DE serovar originally isolated from a patient in Seattle (3), were studied. Chlamydiae were grown by inoculating 75-cm² flasks containing confluent McCoy cell monolayers with stock preparations of each serovar. After 2 h at 37°C in a shaker-incubator, the flask were overlaid with 50 ml of CMGA-HEPES with cycloheximide. The flasks were then incubated at 37°C for 42 to 44 h, and the infected cells were subsequently removed by the addition of 3-mm diameter
sterile glass beads (VWR Scientific, San Francisco, Calif.). After sonication of the cells (30 W for 45 s at 4°C), chlamydia
EB were partially purified by differential centrifugation
(500 × g for 15 min at 4°C, 10,000 × g for 60 min at 4°C, and
500 × g for 15 min at 4°C) and subsequent centrifugation
through 35% Renografin-76 (66% meglumine diatrizoate and
10% diatrizoate sodium; Squibb) at 40,000 × g for 60 min at
4°C (5, 11). The resulting pellet was washed and suspended
in phosphate-buffered saline (PBS) and stored at −70°C in
0.2-ml portions.

Partially purified EB were quantitated by a modification of
the original method described by Furness et al. (9). McCoy
cell monolayers were prepared on 12-mm diameter cover
slips in glass vials (19 by 55 mm; Johns Scientific, Toronto,
Canada) and inoculated with serial dilutions (ranging from 1 × 10⁻³ to 2 × 10⁻⁴) of a representative portion of chlamydiae.
The vials were then centrifuged at 3,000 × g for 60 min at
37°C, and the monolayers were overlaid with 1 ml of
CMGA-HEPES with cycloheximide. After incubation at
37°C for 48 h (L2 serovar) or 60 h (DE serovar), the cells
were fixed with alcohol–Formalin and methanol. The cover
slips were stained with Jones iodine, then removed from the
vials, mounted on slides, and examined microscopically at
100× magnification or, if necessary, 400× magnification.
The number of inclusions was counted at each serial dilu-
tion, and an average number of inclusion-forming units per
milliliter of the original portion was calculated.

PMNs and plasma. Human PMNs and plasma were ob-
tained from the same normal, healthy volunteers. Serum
microimmunofluorescence, as originally described by Wang
et al. (23), and immunoblotting (17) were performed by
Robert C. Brunham, Division of Infectious Disease, Depart-
ments of Medicine and Medical Microbiology, University of
Manitoba, Winnipeg, Canada. All donors were shown to be
negative by microimmunofluorescence (titer ≤ 1:8) for spe-
cific serum immunoglobulin G, immunoglobulin M, and
immunoglobulin A antibodies against C. trachomatis. As
previously observed by others (17), immunoblotting of all
sera revealed nonspecific binding to the major outer mem-
brane protein of L2, D, and J serovars of C. trachomatis.
However, there was no other more specific binding of donor
sera to chlamydial macromolecules detected by this tech-
nique.

Venous blood was collected in heparin (0.25 U/ml of
blood). Plasma was obtained for immediate use by centrifu-
gation of a 10-ml portion of heparinized blood at 500 × g for
10 min at 4°C. Homologous PMNs were collected from the
remaining blood by dextran sedimentation and were purified
by centrifugation through a Ficoll-Hypaque (Litton Bionet-
ics, Kensington, Md.) gradient followed by hypotonic lysis
to eliminate erythrocytes (4). PMNs were suspended in 1 ml
of Hanks balanced salt solution containing 0.1% gelatin and
quantitated with a Sysmex Microcellcounter CC-110 (TOA
Medical Electronics Co., Ltd., Carson, Calif.). Greater than
99% viability of PMNs was confirmed by trypan blue exclu-
sion (15).

Chemotaxant preparations. Various concentrations of
freshly thawed chlamydial EB were added to 0.5-ml portions
of plasma. The preparations were placed in a shaker-
incubator for 2 h at 37°C and then heated to 56°C for 30 min
in a water bath before use in the chemotaxis assay and C5a
des-arginine assays. Chemotaxant preparations of plasma
alone, EB in PBS, EB in plasma preheated to 56°C for 30 min,
and zymosan (Sigma)-activated plasma (25 mg of
zymosan per ml of plasma) were prepared and handled in a
similar fashion.

Chemotaxis assay. Chemotaxis under agarose was per-
formed as originally described by Nelson et al. (16) and as
later modified by Orr and Ward (19). Briefly, Type II arginine
(Sigma) was dissolved in distilled water (by heating in a
boiling water bath) to a concentration of 1.5 g of arginine per
100 ml of water. After cooling to 48°C, the arginine solution
was mixed with an equal volume of warm minimal essential
medium with Earle salts, 26 mM sodium bicarbonate, and
2 mM L-glutamine (GIBCO). The minimal essential medium
was also supplemented with 50 mM Tris buffer and 0.4%
bovine serum albumin (Sigma) before mixture. Approxi-
mately 6 ml of the final arginine medium was poured into
Falcon tissue culture dishes (60 by 15 mm; Becton Dickin-
on) and allowed to harden. Six series of three wells
were cut out of the agarose in a radial fashion with a
Plexiglas template and stainless steel punch. The outer well
of each series received 10 μl of the appropriate chemoat-
ttractant preparation, and the inner well of each series
received 10 μl of Hanks balanced salt solution as a control,
nonchemoattractant buffer. The agarose plates were then
incubated in 5% CO₂ at 37°C for 1 h. After the center well of
each series received 10 μl of Hanks balanced salt solution
with 0.1% gelatin containing 2.5 × 10⁷ PMNs per ml, the
plates were reincubated in 5% CO₂ at 37°C for an additional
2 h. The plates were then fixed with 2.5% glutaraldehyde
overnight, the agarose was removed, and the cells were
stained with Wright’s stain. Chemotaxis was assessed by
examining the plates on an Olympus SH-MPS microscope
with a projector screen. Movement of PMNs away from the
center well was then measured on a centimeter scale as the
linear distance between the original margin of the well and
the leading edge of the PMNs.

To account for the random movement of PMNs, the
chemotactic differential (CD) was calculated for each series
of three wells. This was the distance of PMN migration
towards the chemotaxant well minus the random move-
ment of PMNs in the direction of the inner control buffer
well. For each chemotaxant preparation, a total of 18
series of three wells (three agarose plates) was observed,
and the mean CD was calculated.

C5a des Arg assay. Human complement 125I-labeled C5a
des-arginine (C5a des Arg) radioimmunoassay kits were
employed to measure C5a des Arg levels in all chemoattract-
ant preparations used in the chemotaxis assay. These kits
were provided as a gift by the Upjohn Co., Kalamazoo,
Mich. C5a des Arg is the degradation product of C5a and is
formed after C5a is rapidly acted upon by a carboxypeptidase-N
enzyme (anaphylatoxin inactivator) that is found in normal plasma and serum (1). C5a is the small
fragment of C5 that is released into the fluid phase when C5
is cleaved as a result of alternative or classical complement
pathway activation. C5a is also the most potent chemotactic
factor for PMNs in the complement cascade (8).

Statistical comparisons. All statistical comparisons
were done by a two-tailed Student t test.

RESULTS

Chemotaxis assay. The chemotaxis assay results for three
healthy C. trachomatis antibody-negative donors are shown
in Table 1. When plasma that was preincubated for 2 h with
L2 EB was used as the chemoattractant, the mean CD was
significantly greater than when (i) plasma alone, (ii) L2 EB
incubated in preheated plasma (56°C for 30 min), or (iii) L2
EB in PBS was used as the chemoattractant. The difference
was significant (P < 0.005) for each donor. Similarly, for the
one donor in whom DE chlamydial EB were studied, the
mean CD was also significantly greater (P < 0.005) for plasma preincubated with DE EB than for plasma alone or DE EB in PBS. Zymosan-activated plasma was included as a positive comparison. Zymosan is a potent activator of the alternative complement pathway.

To ensure that the observed chemotaxigenic effect of *C. trachomatis* upon normal plasma was due to specifically the presence of EB and not contamination of the final suspension by nongeneric material (such as endotoxin or cell fragments) during the cell culture and purification process, control suspensions were also tested in the chemotaxis assay of two of the donors. These control suspensions were prepared by processing uninfected McCoy cell monolayers under the same conditions and techniques as required for the growth and partial purification of *C. trachomatis* EB. Preincubation of plasma with volumes of the control suspension equivalent to the volumes used of the EB preparations resulted in no significantly greater CD than when plasma alone or EB in PBS were used as chemoattractants.

**CS5a des Arg assay.** Chemoattractant preparations used in the chemotaxis experiment were assayed for CS5a des Arg levels (Table 2). Plasma preincubated with L2 EB or DE EB demonstrated much higher CS5a des Arg levels than did plasma alone or L2 EB in preheated plasma. In the latter two chemoattractant preparations, in fact, CS5a des Arg levels were undetectable (<4.0 ng per ml) for all three donors. Plasma preincubated with the control suspension was also assayed for CS5a des Arg for the two donors in whom this was studied. In Donor 1, there was no detectable CS5a des Arg, while in Donor 3 the level was low but detectable at 8.31 ± 1.99 ng per ml. This level, however, was significantly lower (P < 0.001) than that measured in plasma preincubated with L2 EB.

Although not shown in Table 2, CS5a des Arg levels were undetectable in the chemoattractant mixtures prepared by incubating L2 EB in PBS. The CS5a des Arg levels measured in zymosan-activated plasma are shown for comparison.

**DISCUSSION**

We have shown in vitro that EB of *C. trachomatis* exert a chemotaxigenic effect upon normal human plasma. As this effect was abolished by preheating plasma to 56°C and since high CS5a des Arg levels were detected in plasma preincubated with chlamydial EB, we conclude that the PMN chemotaxis observed was at least in part related to activation of the complement cascade and subsequent generation of the potent chemotaxin CS5a. The mechanism by which EB activate complement is unknown at present, but it should be emphasized that in our study such activation occurred in the absence of specific antibodies to *C. trachomatis*. As was the case with our donor sera, nonspecific binding of immunoglobulins to the major outer membrane protein of *C. trachomatis* has been demonstrated (17), and this interaction could conceivably initiate complement activation. In addition, lipopolysaccharides resembling the endotoxin of gram-negative bacteria are present in the outer membrane of chlamydiae (7, 12, 18). This chlamydial endotoxin may be capable of stimulating the alternative pathway and thus be responsible for the activation of complement.

Since *C. trachomatis* is an obligate intracellular pathogen, individual organisms are probably not exposed to plasma or plasma components during most of their growth cycle. However, in vivo activation of complement may occur when EB, the infective form of *C. trachomatis*, are released from genital epithelial cells into the extracellular environment and perhaps onto the luminal surface of the infected organ. In this regard, it is important to note that significant concentrations of C3 (an integral complement component) and other plasma proteins, including albumin, transferrin, and immunoglobulins, have been detected in human genital tract secretions, such as normal cervical mucus (6, 22). Furthermore, it is felt that release of EB from epithelial cells often results in lysis of the host cell (21). The subsequent disruption of previously intact epithelium may then enhance the passage of plasma or plasma proteins (such as complement components) to the site of infection. Thus, in vivo activation of complement by *C. trachomatis* may be an important stimulus for the migration of PMNs seen in acute chlamydial infection.

The significance of PMNs in acute genital infection caused by *C. trachomatis* is not known at present. The phagocytic role of such cells may be somewhat restricted by the life cycle of chlamydiae in that intracellular organisms are probably protected from ingestion by PMNs. Nonetheless, PMNs may limit infection by phagocytosing and killing released extracellular EB. In addition to host defense, the

**TABLE 2. Complement activation by *C. trachomatis* as assessed by radioimmunoassay for CS5a des Arg**

<table>
<thead>
<tr>
<th>Chemoattractant preparation*</th>
<th>Mean CS5a des Arg (ng/ml) ± SD† in donor:</th>
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<tr>
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<td>1</td>
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<tr>
<td>L2 EB in plasma</td>
<td>171.00 ± 10.64</td>
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<tr>
<td>DE EB in plasma</td>
<td>37.40 ± 15.76</td>
</tr>
<tr>
<td>Plasma alone</td>
<td>&lt;4.00</td>
</tr>
<tr>
<td>L2 EB in Δ plasma</td>
<td>&lt;4.00</td>
</tr>
<tr>
<td>Control in plasma</td>
<td>&lt;4.00</td>
</tr>
<tr>
<td>ZAP†</td>
<td>550.00 ± 25.81</td>
</tr>
</tbody>
</table>

* Concentrations of L2 EB in plasma, preheated (Δ) plasma, and PBS were 5 × 10⁶ inclusion-forming units per ml for Donor 1, 2 × 10⁶ inclusion-forming units per ml for Donor 2, and 3.4 × 10⁶ inclusion-forming units per ml for Donor 3.
† Mean ± standard deviation (SD) of four measurements.
* NT, Not tested.
‡ P < 0.001 for L2 EB in plasma versus control plasma (by the two-tailed Student t test).
† ZAP, Zymosan-activated plasma.

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**TABLE 1. Chemotaxigenic effect of *C. trachomatis* upon normal plasma as assessed by chemotaxis under agarose assay**

<table>
<thead>
<tr>
<th>Chemoattractant preparation*</th>
<th>Mean CD (cm) ± SD† in donor:</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
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<tr>
<td>L2 EB in plasma</td>
<td>6.36 ± 1.23</td>
</tr>
<tr>
<td>DE EB in plasma</td>
<td>0.71 ± 0.65</td>
</tr>
<tr>
<td>Plasma alone</td>
<td>1.12 ± 0.80</td>
</tr>
<tr>
<td>Δ plasma</td>
<td>1.82 ± 0.50</td>
</tr>
<tr>
<td>PBS</td>
<td>0.64 ± 0.90</td>
</tr>
<tr>
<td>Control in plasma</td>
<td>0.22 ± 0.48</td>
</tr>
<tr>
<td>Plasma alone</td>
<td>0.61 ± 0.61</td>
</tr>
<tr>
<td>ZAP†</td>
<td>8.66 ± 1.48</td>
</tr>
</tbody>
</table>

* Concentrations of L2 EB in plasma, preheated (Δ) plasma, and PBS were 5 × 10⁶ inclusion-forming units per ml for Donor 1, 2 × 10⁶ inclusion-forming units per ml for Donor 2, and 3.4 × 10⁶ inclusion-forming units per ml for Donor 3.
† Mean ± standard deviation (SD) of 18 measurements.
* NT, Not tested.
† ZAP, Zymosan-activated plasma.
PMN and other components of the acute inflammatory response may also be important as mediators of the tissue damage that is seen in many chlamydial infections. The consequences of such tissue damage are most striking in acute salpingitis, which may result in tubal infertility or subsequent ectopic pregnancy (20). However, further in vitro study and in vivo experimentation with animal models are required to determine the relative importance and mechanisms of action of the various components of the acute inflammatory response to infection with *C. trachomatis*.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


