The ability of mononuclear cells to coagulate blood in response to Coxiella burnetii

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Summary. Endocarditis is a rather frequent complication of Q fever caused by Coxiella burnetii. We examined the ability of phase I (virulent) or phase II (avirulent) C. burnetii to coagulate blood in the presence of human blood mononuclear cells in vitro. After incubation for 4 h, virulent phase I C. burnetii was an effective stimulant for mononuclear cells. Since this interaction is a potent trigger of blood coagulation through the extrinsic pathway, it could be responsible for the local deposition of fibrin on the surface of infected valves and the development of large vegetations in cases of endocarditis complicating Q fever.

Introduction

Coxiella burnetii, the gram-negative intracellular agent that causes Q fever, is unique among rickettsiae in exhibiting the phenomenon of phase variation. Virulent C. burnetii phase I is isolated from natural infections, whereas the avirulent phase II is derived from serial laboratory passage in eggs or tissue culture (Baca and Paretsky, 1983). C. burnetii is frequently reported to cause chronic endocarditis, which can occur after Q fever in man (Giroud, 1976; Rosman et al., 1977; Constantinidis and Jenkins, 1979; Kimbrough et al., 1981; Palmer and Young, 1982; Tobin et al., 1982). The deposition of fibrin on infected vegetations and the presence of mononuclear cells are typical features in experimental bacterial endocarditis (Durack, 1975). However, the mechanism(s) by which the deposition of fibrin is induced on infected endocardial lesions is not completely understood. It is well known that, when exposed to gram-negative bacteria or their endotoxic lipopolysaccharides, mononuclear cells are capable of triggering blood coagulation through the extrinsic pathway, i.e. they demonstrate procoagulant activity (Lerner et al., 1971; Niemetz and Fani, 1971; Miragliotta et al., 1982; Miragliotta and Fumarola, 1983). We report here studies with mononuclear cells stimulated by C. burnetii to determine whether they produce similar activity which could be responsible for the clinical features of Q fever endocarditis.

Materials and methods

Blood collection and preparative procedures

Blood was taken from healthy donors into trisodium citrate (0.015 M final concentration). Mononuclear cells were isolated by the Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) gradient technique as described by Boyum (1968). Cell preparations were washed three times with citrated Hanks's Balanced Salts Solution (HBSS) and then resuspended to the desired concentration in HBSS without citrate. Of these cells, 20% were monocytes as assessed by cytochemical reactivity for a-naphthyl acetate esterase (Li et al., 1973) and the remainder were lymphocytes. Granulocyte contamination was less than 5%, and the ratio of platelets to leucocytes was less than 1:1 as determined by light microscopy.

Bacteria

C. burnetii strain Nine Mile (phase I and phase II) was obtained from the Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia. Cells were killed by formalin or phenol and purified as described by Kazar et al. (1983). Cells were suspended in pyrogen-free saline at a concentration of $4 \times 10^8$/ml and stored at $-20{}^\circ$C. Bacterial suspensions were adjusted to the desired concentration just before use. Phase I bacteria treated with a mixture of chloroform and methanol 2:1 v:v for 4 h at 37°C were also used in some experiments.

Effect of bacteria on mononuclear cells

The capacity of monocytes to induce blood coagulation in response to bacteria was studied in whole blood and in isolated mononuclear-cell suspensions. Citrated whole
blood was mixed with each of the bacterial preparations (2 × 10^8/ml of whole blood) or with a similar volume of pyrogen-free isotonic saline, or with bacterial culture supernate and then incubated at 37°C in sterile plastic tubes. At 0 h and after 4 h we determined the procoagulant activity of mononuclear cells in whole blood + bacteria mixtures and of mononuclear cells isolated from these mixtures.

**Evaluation of mononuclear cell procoagulant activity**

The coagulative activity of whole blood + bacteria mixtures was assayed by recording the clotting time after the addition of 0.1 ml of 0.025 M CaCl₂ to 0.2 ml of blood (Biggs, 1972; Osterud and Bjorklid, 1982). Similar activity of mononuclear cells either isolated from the blood-bacteria mixtures or incubated with bacteria soon after their isolation from freshly collected blood was measured in the following test system: 0.1 ml of cell suspension, 0.1 ml of plasma substrate, and 0.1 ml of 0.025 M CaCl₂ (Goutner et al., 1975). Plasma substrate was normal plasma (pooled from at least three normal subjects) and plasma from individuals congenitally deficient in factor VII or factor VIII (Istituto Behring, Scoppito, L'Aquila, Italy). All tests were performed in duplicate in plastic tubes.

**Statistical analysis**

The statistical significance of differences between pairs of means from the experiments was evaluated by Student's t test. The result of each experiment was taken as the mean of duplicate determinations.

**Results**

As shown in table I, clotting activity was evident after prolonged incubation of whole blood with *C. burnetii* phase I. Mononuclear cells isolated from whole blood + *C. burnetii* phase I mixtures after incubation for 4 h shortened the recalciﬁcation time of normal plasma. No signiﬁcant difference was observed between the effects of *C. burnetii* phase I (untreated) and phase I delipidised cells. In the same experimental conditions, *C. burnetii* phase II was signiﬁcantly less effective and no effect was detected when supernates from *C. burnetii* preparations and pyrogen-free saline were added to whole blood.

Similar results were observed when *C. burnetii* phase I (untreated), phase I delipidised, and phase II cells were allowed to interact directly with mononuclear cells. Indeed, as shown in table II, *C. burnetii* phase II cells had signiﬁcantly less effect on mononuclear cells than *C. burnetii* phase I (untreated) and phase I delipidised cells. The ﬁgure shows that the coagulation process was dependent on the dose of *C. burnetii* used.

Further characterisation of the process was obtained from experiments with normal plasma, factor VII- and factor VIII-deﬁcient plasma. Table III shows that factor VII-deﬁcient plasma cannot support the phenomenon in the presence of mononuclear cells obtained from the whole blood + *C. burnetii* phase I mixtures.

**Discussion**

The occurrence of endocarditis following Q fever in man has been frequently described in chronic cases of infection, often associated with hepatomegaly and splenomegaly (Kimbrough et al., 1979; Raoult et al., 1986). Morphological studies by Durack (1975) have shown that bacterial endocarditis could be initiated by phagocytosis of circulating bacteria. Indeed, *Streptococcus sanguis* was found inside mononuclear phagocytes present on the vegetations 30 min after its injection intravenously in rabbits. Other bacteria (e.g., *Staphylo-*

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**Table I.** Plasma clotting activity of suspensions of mononuclear cells (5 × 10^3/μl) from mixtures of whole blood + bacteria after incubation for 4 h at 37°C

<table>
<thead>
<tr>
<th>Bacterial preparation</th>
<th>Number of experiments</th>
<th>Recalcification time (s) (mean, SEM)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. burnetii</em> phase I</td>
<td>10</td>
<td>167.8 SEM 14.5</td>
<td></td>
</tr>
<tr>
<td><em>C. burnetii</em> phase I delipidised</td>
<td>10</td>
<td>138.1 SEM 7.0</td>
<td>*N.S.</td>
</tr>
<tr>
<td><em>C. burnetii</em> phase II</td>
<td>10</td>
<td>258.4 SEM 7.1</td>
<td>†p &lt; 0.01</td>
</tr>
<tr>
<td><em>C. burnetii</em> phase I supernate</td>
<td>10</td>
<td>&gt; 360</td>
<td></td>
</tr>
<tr>
<td><em>C. burnetii</em> phase II supernate</td>
<td>10</td>
<td>&gt; 360</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>10</td>
<td>&gt; 360</td>
<td></td>
</tr>
</tbody>
</table>

*C. burnetii* phase I vs *C. burnetii* phase I delipidised cells.
†*C. burnetii* phase I vs *C. burnetii* phase II.
Table II. Plasma clotting activity of mononuclear cells (5 × 10³/µl) after incubation for 4 h at 37°C with bacteria

<table>
<thead>
<tr>
<th>Bacterial preparation</th>
<th>Number of experiments</th>
<th>Plasma recalcification time (s) (mean, SEM)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. burnetii phase I</td>
<td>5</td>
<td>120.0 SEM 2.4</td>
<td></td>
</tr>
<tr>
<td>C. burnetii phase I delipidised cells</td>
<td>5</td>
<td>125.0 SEM 2.5</td>
<td>*N.S.</td>
</tr>
<tr>
<td>C. burnetii phase II</td>
<td>5</td>
<td>270.0 SEM 3.0</td>
<td>†&lt;0.01</td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>&gt;360</td>
<td></td>
</tr>
</tbody>
</table>

*C. burnetii phase I vs C. burnetii phase I delipidised cells.
†C. burnetii phase I vs C. burnetii phase II.

Table III. Characterisation of mononuclear cell (5 × 10³/µl) plasma clotting activity induced by incubation with C. burnetii (2 × 10⁸/ml) for 4 h

<table>
<thead>
<tr>
<th>Bacterial preparation</th>
<th>Number of experiments</th>
<th>Normal plasma</th>
<th>F VIII deficient</th>
<th>F VII deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. burnetii phase I</td>
<td>4</td>
<td>115.0 SEM 4.0</td>
<td>120.0 SEM 2.5</td>
<td>241.0 SEM 8.5</td>
</tr>
<tr>
<td>C. burnetii phase I delipidised cells</td>
<td>4</td>
<td>111.0 SEM 3.8</td>
<td>118.0 SEM 1.8</td>
<td>237.0 SEM 7.0</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>&gt;360</td>
<td>&gt;360</td>
<td>&gt;360</td>
</tr>
</tbody>
</table>

coccus epidermidis) were present directly on the surface of the vegetations and, within 24 h, bacterial colonies appeared surrounded by capsules of fibrin. To define the possible mechanism(s) of local fibrin formation on the surface of infected vegetations, van Ginkel et al. (1979) showed that mononuclear cells stimulated by bacteria generate thromboplastin which activates the coagulation cascade. Drake et al. (1984), using a rabbit model, suggested that a tissue factor produced by infected valves was responsible for fibrin deposition leading to the development of vegetations. We have demonstrated that C. burnetii stimulates human mononuclear cells to produce an important clot-promoting substance and that phase I micro-organisms are stronger inducers of clotting than micro-organisms in phase II. This finding is consistent with the notion that cells of phase I are analogous to smooth bacteria and are more virulent than cells of phase II (Kazar et al., 1974). Since the demonstration of the simultaneous presence in infected vegetations of fibrin and mononuclear phagocytes (Durack, 1975), and the isolation of C. burnetii itself from the aortic valve (Robson and Shimmin, 1959; Kimbrough et al., 1981), it appeared reasonable that tissue factor production might represent an important step in
local fibrin formation on the surface of infected vegetations. On the other hand, an inhibitory effect of warfarin treatment on the development of experimental *S. epidermidis* endocarditis has been described (Thorig et al., 1977). This is in agreement with our hypothesis, because warfarin interferes with factors II, VII, IX, and X of the blood coagulation sequence which are involved in the pathway activated by the mononuclear cells. Furthermore, in the original observations of the gross pathology of Q fever by Derrick (1937) and Burnet and Freeman (1937), splenomegaly was described. This condition involves a considerable increase in the number of mononuclear cells (i.e., lymphocytes and macrophages). Finally, in our experiments no significant difference was demonstrated between *C. burnetii* phase I (untreated) and phase I delipidised cells in their capacity to induce clotting. Treatment of bacterial cells with chloroform-methanol removes lipids and these results indicate that lipid components are not responsible for this activity. Our data strongly support the hypothesis that the expression of clotting by blood mononuclear cells in response to the presence of *C. burnetii* might contribute to the fibrin deposition and enlargement of the vegetation seen in Q fever endocarditis.

**REFERENCES**


