Virus-associated apoptosis of blood neutrophils as a risk factor for invasive meningococcal disease

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ABSTRACT

Aims To quantify a range of haematological indicators of viral infection (leucocyte apoptosis, cytopenia of normal lymphocytes, reactive lymphocyte increase, neutropenia) in patients with recent onset invasive meningococcal disease (IMD), with a view to test the association of viral infection with IMD and identify possible haematological risk factors for its development.

Subjects and methods 88 patients with recent onset IMD, classified on clinical severity as fatal (n=14), septic shock survived (n=26) and no shock (n=48), and 50 healthy controls were studied. Blood film microscopy and leucocyte counts were used to quantify the virus-associated indicators. Cocci-containing neutrophils were also quantified.

Results All viral parameters were significantly more frequent or higher in patients than controls, with leucocyte apoptosis found only in the patients. A significant gradient in accord with clinical severity was found for neutrophil and lymphocyte apoptosis, neutropenia and cocci-containing neutrophils. Crucially, apoptotic neutrophils did not contain cocci, and cocci-containing neutrophils were not apoptotic.

Conclusions The correlation between magnitude of neutrophil apoptosis and severity of IMD suggests a cause–effect relationship. We propose that neutrophil apoptosis is more likely a facilitator rather than an effect of IMD for these reasons: (1) apoptotic neutrophils did not contain cocci and cocci-containing neutrophils were not apoptotic, (2) leucocyte apoptosis is a recognised viral effect and (3) Neisseria meningitidis is incapable of producing a Panton–Valentine type leucocidin. The lymphocyte apoptosis which accompanies neutrophil death may contribute to risk by impairing the generation of microbialic antibody. Leucocyte apoptosis is a morphological expression of viral immunosuppression and, we suggest, is a likely contributor to a range of viral effects.

INTRODUCTION

A variety of factors affecting nasopharyngeal mucosal integrity, systemic immunity and the biology of the organism itself are risk factors for recent onset invasive meningococcal disease (IMD). These include: preceding viral infection, especially influenza A,1 mycoplasma infection,2 cigarette smoking, exposure to cigarette smoke or smokers, exposure to construction site dust,3–5 and deficiencies in systemic immunity relating to serum bactericidal antibody to meningococcus,6 mannose-binding lectin8,9 and more rarely, deficiencies in components of the terminal pathway of complement activation or properdin.10 A single risk factor may operate through several mechanisms; for example, the fact that postinfluenzal spikes of IMD are in general not accompanied by increased meningococcus carriage has suggested virus-induced neutrophil dysfunction as a contributor to risk.1 In addition, viruses have the capacity to enhance mucosal adhesiveness for bacteria11–12 or, in the case of influenza A, the capacity of its neuraminidase to cleave the sialic acid-containing capsular polysaccharides of Neisseria meningitidis serogroups B, C and W135 to enhance adhesiveness to epithelial surfaces.13 The innate capacity of the bacterial strain for invasiveness through epithelium is also significant in pathogenesis of IMD.14

We quantified a range of haematological indicators of viral infection with the aims of substantiating, from a novel perspective, the linkage between viral infection and IMD and of identifying possible haematological risk factors for its development. Leucocyte apoptosis was assessed, in addition to traditional leucocytic indicators of viral infection:15 reactive lymphocyte increase, cytopenia of normal lymphocytes (lymphocytopenia) and neutropenia. Apoptosis of leucocytes, including reactive lymphocytes, is a feature of the blood film in viral infections, including infectious mononucleosis (IM)16–19 (figures 2 and 3), neonatal herpes simplex viraemia17 20 (figure 1), rubella, measles17 and influenza A infection (Kerwick A-M, unpublished), and is the basis for the macrophage phenomenon in earlobe blood.16 21 Monocytes/macrophages containing degenerate leucocytes (figure 3, ie, a process of haemophagocytosis), on account of their size, accumulate in the rich capillary bed of the earlobe, with simultaneous venous blood films showing few or none of these cells. The occurrence of apoptotic lymphocytes in the blood film in IM correlates with the upregulation of the Fas-receptor/Fas-ligand (Fas-L) system on the primed T lymphocyte population,22 and its propensity to substantial death in culture.23–25

SUBJECTS AND METHODS

Patients and controls

A total of 88 patients with recent onset IMD notified to the Queensland Health Public Health Microbial Reference Laboratory between October 2003 and October 2005 were studied. In all cases, cultures were positive from blood and/or cerebrospinal fluid, except for one patient, with culture positive only from the anterior chamber of an affected eye (table 1). Patients were classified into three groups on clinical severity: fatal, septic shock survived (hypotension with or without poor capillary refill) and no shock. There was a trend, but not significant, toward over-representation of
younger patients in the fatal group. There was a significant over-representation of serogroup C in the fatal group. The control group comprised asymptomatic individuals with no clinical history of infection for at least 3 weeks prior to test. Children in this group were elective admissions for clean surgical procedures such as hernia repair.

**Blood film microscopy**

Wright’s stained blood films prepared immediately on receipt of EDTA-anticoagulated venous blood were used. Films were examined with an oil immersion ×60 planapochromat objective in the optimal part (one cell thick, evenly spread), working methodically in zigzag fashion from one edge to the other to end with a total of about 400–1000 leucocytes examined, depending on the leucocyte count (which took about 5 min). Large granular lymphocytes were counted as normal lymphocytes.

Apoptosis (figures 1–3) was identified by condensation, obliteration of fine structure and glassy homogenisation of nuclear chromatin, leaching of chromatin stain into the cytoplasm, nuclear pyknosis and disintegration, and shrinkage of nucleus and cell as a whole. In advanced degeneration, especially of neutrophils, the chromatin may lose colouration and stain grey (figure 3). Some degenerate lymphocytes were recognisable as reactive or large granular types. These changes were readily distinguishable from anticoagulant/storage artefact.26

Established age-appropriate normal ranges for leucocyte counts15 17 were applied. Age-appropriate upper limits of normal for reactive lymphocytes were taken from our data for the controls as: for those <5 years, <0.09×10⁹/L (<90 per mm³) and for those ≥5 years, <0.06×10⁹/L (<60 per mm³). Apoptotic leucocytes and cocci-containing neutrophils (figure 4) were quantified both as a count per mm³ and, less precisely though nevertheless of some practical utility, as the proportion of subjects positive for the feature. This latter mode of expression of results, being only semiquantitative, was not subjected to statistical analysis. Cocci-containing monocytes17 were not seen in this study.

**Statistics**

Proportions were compared by contingency tables analysis, including Armitage–Mantel–Haenzel ordered test between patient groups. Quantitative variables were compared by Kruskal–Wallis non-parametric one-way analysis (χ² statistics).

**RESULTS**

All viral parameters were significantly greater or more frequent in patients than in controls, with leucocyte apoptosis found only in the patients (table 2). The following characteristics showed a significant gradient in accord with clinical severity: apoptotic leucocytes as a whole (which included on average across all patient groups, 14.4% of dead leucocytes of unidentified type), apoptotic neutrophils, apoptotic lymphocytes,

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**Figure 1** Apoptotic neutrophil (left), apoptotic lymphocyte (right), blood film, neonatal herpes simplex viraemia. Reproduced, with permission, from Smith H. Diagnosis in Paediatric Haematology 1996. Churchill-Livingstone ©, Elsevier.

**Figure 2** Degenerate neutrophil adherent to monocyte, earlobe blood film, infectious mononucleosis. Reproduced, with permission, from Smith H. Diagnosis in Paediatric Haematology 1996. Churchill-Livingstone ©, Elsevier.

**Figure 3** Inclusions of lymphocyte, erythrocyte and neutrophil in advanced degeneration (arrow) in monocyte, earlobe blood film, infectious mononucleosis. Reproduced, with permission, from Smith.17 Churchill-Livingstone ©, Elsevier.
neutropenia and cocci-containing neutrophils. Crucially, apoptotic neutrophils did not contain cocci, and cocci-containing neutrophils were not apoptotic (figure 4). Of the traditional indicators of viral infection, lymphocytopenia was more sensitive than reactive lymphocyte increase or neutropenia, occurring in 77%–79% across all patient groups and in 10% of controls.

**DISCUSSION**

The correlation between magnitude of neutrophil apoptosis and severity of IMD (table 2) suggests a cause–effect relationship. We propose that neutrophil death is a facilitator of IMD rather than an effect for these reasons: (1) Cocci-containing neutrophils were not apoptotic (figure 4) and apoptotic neutrophils did not contain cocci; these observations are in accord with studies suggesting that phagocytosed *Neisseria* sp. can manipulate intracellular processes to enhance the survival of the neutrophil as a safe haven for dissemination of the infection. (2) The late intracellular processes to enhance the survival of the neutrophil, which is well preserved, blood film, invasive meningococcal disease. Courtesy of RG Wells.

**Figure 4** *Neisseria meningitidis* within neutrophil, which is well preserved, blood film, invasive meningococcal disease.Courtesy of RG Wells.
vivo (Smith H, unpublished). Although EBV can infect CD4 and CD8 T lymphocytes in vitro, the expanded EBV-specific CD8 population in IM is not infected; and though Fas is upregulated on this population, inhibition studies show that the Fas system is not required for their apoptosis in culture (compare with IVA above). The cellular toxicity of cytochrome-c released from damaged mitochondria, together with the fact that IM T cells in culture can be rescued from apoptosis by a range of cytokines, has suggested a mitochondrial/cytokine resuscuable mechanism for the cell death. Interleukin-2, as an activator of apoptosis, may have especial relevance, being markedly depleted in apoptosis-prone IM T cells in culture. In neoplastic cells, simplex viremia, the virus can invade and destroy lymphocytes.

The relation between clinical severity and burden of coccicecontaining neutrophils (table 2) accords with the experience that the main determinant of clinical severity in IMD is the magnitude of the bacteraemia.

In the wider context of virus-induced pathology, we suggest that leucocyte apoptosis is a likely contributor to a range of viral effects: (a) Leucocyte death would add to T lymphocyte activation and proliferation as a source for the increase in plasma cytokines to which the malaise and fever of viral infection are attributed. A linkage between a profusion of apoptotic lymphocytes in the blood film in IM and a more severe and protracted illness supports this proposition. (b) Shedding of dehydrogenase resulting from the liver damage and, less so in IM. The high increase in serum levels of soluble CD8 in IM (sCD8 ∼ 102× normal, sCD8 ∼ 1.2× normal) may be attributed to high activation and proliferation of the CD8 population, and to its high death rate in vivo (see above). (e) Apoptosis contributes to depletion of CD4 lymphocytes which, together with depletion of functional macrophages, underlies the cutaneous anergy, as assessed by delayed hypersensitivity testing, which is common and severe in measles, and less so in IM. (f) The high increase in serum levels of soluble CD8 in IM (sCD8 ∼ 102× normal, sCD8 ∼ 1.2× normal) may be attributed to high activation and proliferation of the CD8 population, and to its high death rate in vivo (see above). (g) Leucocyte apoptosis, by stimulating phagocytosis, and activation of macrophages may be significant in the genesis of virus-associated haemophagocytic syndrome.

The model we propose for virus–IMD association may also apply to other viral–bacterial associations, such as postinfluenzal pneumococcal sepsis, and neonatal herpes simplex viremia presenting as bacterial septicaemia.

**Table 2** Leucocyte data, patients and controls

<table>
<thead>
<tr>
<th>Patients</th>
<th>Fatal</th>
<th>Septic shock survived</th>
<th>No shock</th>
<th>Difference between patient groups</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>13/14 (93%)</td>
<td>16/26 (62%)</td>
<td>18/48 (38%)</td>
<td>p&lt;0.0003</td>
<td>0/50</td>
</tr>
<tr>
<td>Per mm³, mean (SD)</td>
<td>89.3 (78.6)</td>
<td>46.6 (80.3)</td>
<td>34.9 (117.6)</td>
<td>p&lt;0.0003</td>
<td>0</td>
</tr>
<tr>
<td>Subjects</td>
<td>9/14 (64%)</td>
<td>13/26 (50%)</td>
<td>16/48 (33%)</td>
<td>p=0.03</td>
<td>0</td>
</tr>
<tr>
<td>Per mm³, mean (SD)</td>
<td>36.5 (47.8)</td>
<td>19.5 (28.3)</td>
<td>9.6 (16.5)</td>
<td>p=0.03</td>
<td>0</td>
</tr>
<tr>
<td>Neutropenia*</td>
<td>5/14 (36%)</td>
<td>2/26 (8%)</td>
<td>1/48 (2%)</td>
<td>p&lt;0.0001</td>
<td>1/50 (2%)</td>
</tr>
<tr>
<td>Neutrophils with cocci</td>
<td>11/14 (79%)</td>
<td>4/26 (15%)</td>
<td>1/48 (2%)</td>
<td>p&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td>Per mm³, mean (SD)</td>
<td>48.6 (43.4)</td>
<td>4.9 (13.0)</td>
<td>0.4 (2.7)</td>
<td>p&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td>Subjects</td>
<td>13/14 (93%)</td>
<td>12/26 (46%)</td>
<td>10/48 (21%)</td>
<td>p&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td>Per mm³, mean (SD)</td>
<td>42.0 (52.7)</td>
<td>17.1 (26.9)</td>
<td>22.01 (107.4)</td>
<td>p&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytopenia</td>
<td>11/14 (79%)</td>
<td>20/26 (77%)</td>
<td>38/48 (79%)</td>
<td>p=0.98</td>
<td>5/50 (10%)</td>
</tr>
<tr>
<td>Reactive lymphocyte increase</td>
<td>5/14 (36%)</td>
<td>9/26 (35%)</td>
<td>9/48 (19%)</td>
<td>p=0.18</td>
<td>4/50 (8%)</td>
</tr>
</tbody>
</table>

*All patients combined versus controls, p<0.001.
†Mean value inflated by one high value outlier; median=0.
‡All patients versus controls, p<0.0001.
§All patients versus controls, p<0.0001.
¶All patients versus controls, p=0.02.

**What the study adds**

Haematological indicators of viral infection substantiate the association of viral infection with invasive meningococcal disease (IMD). Leucocyte apoptosis is a morphological expression of viral immunosuppression and a likely facilitator of IMD: neutrophil death by impairing phagocytic capacity, and lymphocyte death by impairing generation of microbicidal antibody. In the wider context of viral pathology, we suggest that leucocyte apoptosis is a likely contributor to a range of viral effects, including fever and malaise, lupus-type anticoagulants, blood cytopenias, cutaneous anergy, and virus-associated haemophagocytic syndrome.
Haematological indicators of viral infection substantiate the association of viral infection with invasive meningococcal disease (IMD).

Leucocyte apoptosis is a morphological expression of viral immunosuppression and a likely facilitator of IMD by impairing two arms of the immune system: phagocytic capacity (neutrophil death) and generation of microbicidal antibody (lymphocyte death).

Leucocyte apoptosis is a likely contributor to a range of viral effects.

The virus–meningococcal link may be a model for other viral–bacterial associations.

We suggest that virus-associated leucocyte apoptosis merits more detailed analysis.

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Contributors HS: conception, design and coordination; writing of manuscript, which was approved by all coauthors. SLR: blood film microscopy. HVS: meningococcal identification and serogrouping. DG: critical commentary on immunological aspects of the phenomenon. VS: statistical analysis. JAS: sourcing and critical assessment of references, presentation of manuscript.

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Competing interests None.

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