Lactoferrin, Lysozyme, and $\beta_2$-Microglobulin in Cerebrospinal Fluid

Elevated Levels in Patients with Acute Cerebrovascular Lesions as Indices of Inflammation

ANDREAS TERENT, M.D., ROGER HÄLLGREN, M.D., P. VENGE, M.D., AND KJELL BERGSTROM, M.D.

SUMMARY Serial determinations of $\beta_2$-microglobulin, lactoferrin and lysozyme in CSF were performed in 14 patients with acute cerebrovascular lesions. Marked elevations were noted in patients with cerebral bleeding or hemorrhagic infarction. Patients with infarction without signs of bleeding or with cerebrovascular lesions undetectable by computed tomography also had an increase in these proteins. The increases in CSF of $\beta_2$-microglobulin, lactoferrin and lysozyme could not be explained by a damaged blood-brain barrier but was believed to be a local product of the central nervous system. Peak levels of lactoferrin and lysozyme were noted on day 2–3 after onset of symptoms. Lactoferrin then declined while lysozyme remained elevated for another few days. $\beta_2$-microglobulin gradually increased reaching peak levels on day 4–5 and remained elevated even 2 weeks after the onset of symptoms. We suggest that the increases of lactoferrin, lysozyme and $\beta_2$-microglobulin reflect various inflammatory reactions mediated by granulocytes, macrophages and lymphocytes, respectively.

THE APPEARANCE after a cerebrovascular lesion of various myeloid or lymphoid cell types in the damaged brain areas and in the cerebrospinal fluid (CSF) indicates a local cerebral inflammatory reaction. A prominent feature of an inflammatory reaction is the extracellular release of lyosomal proteins such as lysozyme and lactoferrin from activated macrophages and neutrophils. $\beta_2$-Microglobulin is located in both the primary and secondary neutrophil granules, whereas lactoferrin is found only in the secondary granules. In addition, lysozyme is found in monocytes and macrophages. $\beta_2$-Microglobulin, which appears on the surface of most cell types as part of the histocompatibility antigens, seems in certain situations to reflect lymphocyte activity. Various cell lines produce $\beta_2$-microglobulin in vitro but lymphoid cell lines especially secrete appreciable quantities, elevated levels of this protein in various body com-
parts in certain inflammatory disorders have been explained by a local secretion from lymphoid infiltrates or by an increased lymphocyte mass and/or turnover. In view of the various cellular distributions of lysozyme, lactoferrin and β2-microglobulin, simultaneous measurement of these proteins in the CSF of patients with acute stroke might be expected to yield information on the type and magnitude of the inflammatory response taking place in the brain or the leptomeninges. An attempt to elucidate the kinetics of the inflammatory cellular reactions was made by serial determinations of these proteins in the CSF of patients with acute cerebrovascular disorders.

Materials and Methods

This study comprised 14 patients — 4 women and 10 men — with acute cerebrovascular disorders. The mean age was 65.3 years (range 58–83) years. The diagnoses were based on history, neurological examination, CT-finding and spectrophotometric and microscopic analyses of CSF. In all patients presented in table 1, clinical signs of focal disturbance of cerebral function had rapidly developed with no apparent cause other than vascular. One patient (No. 12) had several short-lived attacks during the first 2 days after admission with complete remission inbetween. Three other patients (Nos. 7, 11, 13) showed complete recovery during the first week at the hospital. The other patients had persistent neurological symptoms. Three died within 3 weeks and their cerebrovascular lesions were documented by autopsy. Patient No. 2 died on day 20 following admission in cardiogenic shock with massive pulmonary embolism. The autopsy showed a small hemorrhage in vermis cerebelli. Patients Nos. 5 and 6 died on days 18 and 21, respectively, as a consequence of their cerebral infarcts.

The first examination took place as soon as possible after admission to the hospital and usually within 10 hours after the onset of symptoms; the second examination took place on the second or third day; the third after 4 or 5 days and the fourth after 10 to 15 days. On each occasion neurological examination was followed by CT scan and collection of CSF by lumbar puncture. No clinical or roentgenological signs of increased intracranial pressure were present at the time of the second, third or fourth examinations. No reactions to the lumbar punctures were observed.

Complete CT examination was performed before and after intravenous contrast infusion using an EM1 1010 head scanner. The contrast material, Isoopaque Cerebral® (Nyegaard, Oslo), was administered at a dose of 1 ml/kg body weight. The extent of the cerebral lesion was calculated as the largest diameter found on CT scanning of the low attenuation area for infarcts and the high attenuation area for hemorrhages. According to the clinical symptoms 12 patients had supratentorial lesions. Patient No. 2 had signs of intracerebellar hemorrhage and patient No. 14 had multiple signs of a brain stem lesion which was not visualized on the CT scan. CSF (about 8 ml) was withdrawn at each examination and was subjected to the following analyses:

White blood cell (WBC), differential leukocyte and erythrocyte (RBC) counts with a Fuchs-Rosenthal counting chamber.

Spectrophotometry, which was started with

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Lesion type</th>
<th>RBC count (10^6/1)</th>
<th>WBC count (10^3/1)</th>
<th>Fibrinogen (mg/1)</th>
<th>Albumin (mg/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Haemorrhage</td>
<td>2.6</td>
<td>1</td>
<td>90</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Haemorrhage</td>
<td>4.5</td>
<td>960,000</td>
<td>101</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>Haemorrhagic infarct</td>
<td>11.3</td>
<td>886</td>
<td>91</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>Infarct</td>
<td>3.8</td>
<td>68</td>
<td>91</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
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<td>6.4</td>
<td>580</td>
<td>38</td>
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</tr>
<tr>
<td>6</td>
<td>Infarct</td>
<td>11.6</td>
<td>346</td>
<td>335</td>
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</tr>
<tr>
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<td>Infarct</td>
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<td>20</td>
<td>57</td>
<td>1.7</td>
</tr>
<tr>
<td>8</td>
<td>Infarct</td>
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<td>3,033</td>
<td>20</td>
<td>1.7</td>
</tr>
<tr>
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<td>48</td>
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<tr>
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<td>1,335</td>
<td>119</td>
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<tr>
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<td>640</td>
<td>54</td>
<td>1.7</td>
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<tr>
<td>12</td>
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<td>—</td>
<td>2</td>
<td>86</td>
<td>1.5</td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>—</td>
<td>198</td>
<td>28</td>
<td>1.0</td>
</tr>
<tr>
<td>14</td>
<td>Negative</td>
<td>—</td>
<td>56</td>
<td>31</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Abbreviations: Adm. = at admission, Max = peak level.
screening of the absorbance at 415 nm. It was assumed that the specimen contained significant amounts of hemoglobin or hemoglobin breakdown products if the absorbance exceeded 0.040 and, in that case, the spectrum of absorbance was completely scanned.

Albumin was determined by a nephelometric technique. The coefficient of variation of the assay was 5 percent.

Lactoferrin and lysozyme were assayed in duplicate with radioimmunoassays using antibodies coupled to CNBr-activated Sephadex and 125I-labelled lactoferrin or lysozyme. The coefficients of variation for the methods were 6 and 7 percent, respectively.

β2-microglobulin was assayed in duplicate using the Phadebas β2-micro test (Pharmacia AB, Uppsala, Sweden). The assay procedure was carried out according to the manufacturers' instructions. The coefficient of variation of the assay was 6 percent.

Serum levels of lactoferrin, lysozyme and β2-microglobulin were measured by radioimmunoassays as above. The normal serum ranges of the proteins as estimated on 35 healthy blood donors are listed in table 2.

The reference group for CSF values was 33 patients (15 women and 18 men) with a mean age 45.4 (range 20–77) years. These individuals were admitted for neurological examination because of diffuse symptoms of long duration (headache, dizziness, pain). No abnormalities were found by clinical and laboratory examinations and the patients were considered healthy. CSF specimens were analysed for RBC and WBC, lactoferrin, lysozyme and β2-microglobulin.

**Results**

In table 2 the CSF concentrations of lactoferrin, lysozyme, β2-microglobulin and albumin in the patient reference group are shown. Based on the mean CSF concentration and the mean normal serum concentration for these proteins (calculated as geometric means due to the log-normal distribution of the proteins) the mean "normal" CSF: serum ratio for lactoferrin, lysozyme, β2-microglobulin and albumin were calculated to 0.015, 0.022, 0.688 and 0.004, respectively.

Serial determinations of the CSF-levels of lactoferrin, lysozyme, β2-microglobulin and albumin in patients with cerebrovascular lesions are presented for the group in figure 1 and for some individual patients in figure 2. The initial level of lactoferrin obtained during the first day after onset of symptoms was elevated above the upper reference range in 2/3 of the patients with cerebral hemorrhage and in 3/8 of the patients with cerebral infarction verified by CT (table 1). When calculated for all patients lactoferrin increased significantly (p < 0.01) on days 2–3 after the onset of symptoms and the individual increase was on average 92 percent compared to the initial level. Eight of 14 patients had peak levels of lactoferrin above the reference range (table 1). Lactoferrin gradually declined from day 2–3 and reached a mean level slightly below the initial one on days 12–14.

The initial levels of lysozyme were elevated in all patients with cerebral hemorrhage and in 3 with infarction. Lysozyme increased significantly (p < 0.01) during the early course and the individual increase averaged 198 percent. Peak levels occurring on days 3 and 5 were above the control range in 12/14 patients (table 1 and figure 1). β2-microglobulin was elevated at admission in 11/14 patients (table 1) and continued to increase significantly (p < 0.001) in all individuals reaching peak levels on day 5 or days 12–14. The peak individual β2-microglobulin levels were on average 40 percent higher than the initial levels. The patients with cerebral bleeding or hemorrhagic infarction showed a tendency for higher levels of lactoferrin, lysozyme and β2-microglobulin than the patients with infarction without signs of bleeding. Conversely, the patients with symptoms but no visible signs of cerebral lesions had the lowest levels of these proteins. However, no statistical relationship was found between the extent of lesion as estimated by CT and the peak levels of any of the proteins. A significant correlation (r = 0.58, p < 0.001) was found between CSF-lactoferrin and CSF-lysozyme (fig. 3), while no correlation was found between CSF-β2-microglobulin and lactoferrin or lysozyme (r = 0.08 and 0.12, respectively).

Although increments of CSF-albumin were observed in individual patients (table 1 and fig. 2) no significant changes (p > 0.05) of CSF-albumin were noted during the observation period when calculated on all patients with cerebrovascular lesions (fig. 1). Analyses of the individual patients indicated that the time of the rise of CSF-albumin did not coincide with the rise of lactoferrin and lysozyme (fig. 2). The relative changes of CSF-lactoferrin and lysozyme

**Table 2. The Mean Levels of Lactoferrin, Lysozyme, β2-Microglobulin and Albumin in CSF of the Reference Patients and in Serum of Healthy Individuals.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Lactoferrin μg/l</th>
<th>Lysozyme μg/l</th>
<th>β2-Microglobulin mg/l</th>
<th>Albumin mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>33</td>
<td>5.7 (2.9–14)</td>
<td>38 (21–69)</td>
<td>1.1 (0.7–1.6)</td>
<td>172 (93–292)</td>
</tr>
<tr>
<td>Serum</td>
<td>35</td>
<td>389 (190–645)</td>
<td>1714 (984–2987)</td>
<td>1.6 (1.1–2.4)</td>
<td>48 (40–64)</td>
</tr>
<tr>
<td>Mean ratio (CSF:serum)</td>
<td>0.015</td>
<td>0.022</td>
<td>0.688</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

*Geometric
†Geometric
were also more pronounced than those of CSF-albumin. The more modest increments of β2-microglobulin were in individual cases quite similar to those of albumin. (fig. 2)

In 6 patients with cerebral infarction CSF and serum samples were collected at the same time. Although slight but inconstant variations of serum levels of lysozyme, lactoferrin or β2-microglobulin were observed in individual patients, no increments above the control range were found. Neither did the increase of the serum proteins coincide with those in CSF.

Discussion

The origin of lactoferrin, lysozyme or β2-microglobulin in CSF in individuals without signs of CNS-disorder is obscure. Based on the assumption that albumin (m.w. 69,000 dalton) passively penetrates from blood into CSF, lactoferrin would, from its molecular weight (76,000 dalton) without taking into consideration its electrical charge or molecular size, be expected to have about the same CSF:serum ratio as albumin. However, the mean ratio was about 4 times higher for lactoferrin (table 2). The lysozyme CSF:serum ratio was about 7 times higher and the β2-microglobulin ratio about 170 times higher than that for albumin (table 2). The low molecular weights of lysozyme (15,000 dalton) and β2-microglobulin (m.w. 11,800 dalton) would presumably allow for an increased filtration of these proteins from plasma to CSF and thus explain the CSF-level of lysozyme, but it hardly would explain that of β2-microglobulin. These calculations, then, might suggest either an active transport of at least lactoferrin and β2-microglobulin into the CSF or, more likely, a local production of these proteins inside, i.e., on the brain side, of the blood-brain barrier.

Under normal conditions the serum levels of lactoferrin mainly reflect the turnover of neutrophils,16,20 while those of lysozyme reflect the turnover of both neutrophils and macrophages.20,21 In analogy, the kinetics of such cells, if present in the central nervous system, might be reflected by the CSF-levels of

Figure 1. Serial determinations of the CSF-concentrations of lactoferrin, lysozyme, β2-microglobulin and albumin (geometric means ± SEM) in 14 patients with acute cerebrovascular lesions. The patients are illustrated as 3 groups based on the lesion type found at CT. Closed circles (●) indicate patients Nos 1–3 with cerebral bleeding or hemorrhagic infarction, open circles (○) indicate patients Nos. 4–11 with cerebral infarction and △ means patients Nos. 12-14 with negative CT-findings. Significant increments of the various proteins compared with the levels in the first CSF specimen, as tested by Student's paired t-test on the whole material, are shown (* p < 0.05, ** = p < 0.01, *** = p < 0.001). The broken lines represent the geometric means of the CSF-proteins for the whole group. The shadowed areas represent the ranges of the reference group (table 2).
lactoferrin and lysozyme. At activation of neutrophils and macrophages by inflammatory agents, extracellular release of their lysosomal constituents may occur leading to increased CSF-levels. The basal CSF-levels of β₂-microglobulin presumably reflect the total cell mass of the central nervous system since β₂-microglobulin is produced by all nucleated cells investigated. However, elevated serum-β₂-microglobulin — if not due to impaired renal function — is often connected with lympho-proliferative disorders.

**FIGURE 2.** Serial determinations of lactoferrin, lysozyme, β₂-microglobulin and albumin in CSF of individual patients with hemorrhagic infarct (patient No. 3), with non-hemorrhagic infarct (patients Nos. 5 and 9) and with transitory ischemic attacks (patient No. 12).

**FIGURE 3.** Lactoferrin versus lysozyme concentrations in CSF-specimens obtained from 14 sequentially-studied patients with acute cerebrovascular lesions. The solid line is the regression line (log lysozyme = 0.475 log lactoferrin + 1.469); r = 0.58, p < 0.001. The equation of the regression line for the reference groups of patients is log lysozyme = 0.141 lactoferrin + 1.494 (r = 0.22, p > 0.05).
and is supposed to reflect an increased lymphocyte activity and/or turnover.\textsuperscript{19, 24} Thus, previously reported elevated CSF-$\beta_2$-microglobulin in infectious brain or meningeal disorders\textsuperscript{25-27} might be due to an increased lymphocyte mass located within the central nervous system.

The early appearance of inflammatory cells in CSF after cerebral hemorrhage or contusion has long been considered to reflect a leptomeningeal cell reaction taking place when blood infiltrates the CSF. The present results extend our knowledge in this area, since we could detect during the early course after acute cerebrovascular lesions increased amounts of neutrophil and macrophage products without the gross appearance of such cells in the cerebrospinal fluid. Although those patients who had cerebrovascular lesions with bleeding had the highest elevations, we also observed increasing levels of lysozyme and lactoferrin in patients with infarction without CT signs of hemorrhage. The increase of lactoferrin occurred in most situations on the second-third days after onset of symptoms and then declined to normal levels. Since lactoferrin is a constituent of secondary neutrophil granules\* this observation is interpreted to mean that neutrophils derived from the peripheral circulation are transiently activated during the early course of cerebral lesion.

Lysozyme levels, which also increased early after cerebral lesions, significantly correlated to those of lactoferrin, suggesting that these granular proteins are mainly released by common mechanisms. However, lysozyme remained elevated when lactoferrin started to decline 4 to 6 days from onset of symptoms. Since lysozyme is present not only in neutrophil granules\* but also in macrophages, this difference in CSF-patterns between lysozyme and lactoferrin might be explained by the later appearance of activated macrophages in damaged brain areas. The lack of correlation between CSF-levels of lysozyme and lactoferrin in the reference group of patients (legend of fig. 3) also suggest differences in cellular origin for these proteins.

$\beta_2$-microglobulin behaved in a fashion quite different from lysozyme and lactoferrin. After acute stroke due to bleeding or infarction we noted over a 5 day period a steady increase of CSF-$\beta_2$-microglobulin which remained elevated even after 2 weeks. The relative increase was not as prominent as that observed for lactoferrin or lysozyme. This pattern of $\beta_2$-microglobulin changes might reflect a gradually increased lymphocyte mass and/or activity within the damaged brain area. An alternative explanation is that $\beta_2$-microglobulin was released from necrotic brain tissue or proliferating glial cells. However, the gliosis is not a prominent finding before the fourth week after a cerebrovascular lesion.\textsuperscript{1}

The increments of CSF-albumin observed in individual patients suggest a pathological alteration of the blood-CSF barrier which may contribute to the raised CSF levels of lysosomal proteins and $\beta_2$-microglobulin. However, some available data argue against an increased passive penetration of these proteins through the blood-CSF barrier as the major mechanism behind the elevated CSF-levels in most stroke situations. First, the time sequence of changes of CSF-albumin was not coincidental with that of CSF-lactoferrin or CSF-lysozyme. Second, the increases of the lysosomal proteins not only preceded those of albumin but were also relatively more pronounced. The relatively modest increments of CSF-$\beta_2$-microglobulin, which in individual patients were similar to those of albumin (fig. 2), might, at a cursory glance, be explained by a damaged barrier. However, such a conclusion is less likely assuming that the passive penetration of $\beta_2$-microglobulin from blood to CSF roughly resembles the penetration of albumin. While a minor damage of the barrier should be detectable as increased CSF-albumin levels, due to the high concentration gradient over the blood-brain barrier for albumin, a 2-3 times increased passive penetration of $\beta_2$-microglobulin from blood to CSF may hardly be detectable as significantly raised CSF-concentrations of these proteins, due to the small difference between the serum and CSF concentrations of $\beta_2$-microglobulin (table 2). In order fully to rule out a major influence of altered blood-CSF barrier on the CSF-levels of lactoferrin, lysozyme and $\beta_2$-microglobulin in cerebrovascular lesions, concomitant measurement of the serum levels of these proteins is needed. In some patients serum data were available but there was no increase in the levels of serum proteins measured which could be connected with increments in CSF, favoring the hypothesis of a local production within CNS.

Inflammatory cells releasing their constituents serve as potentially destructive sources in the damaged brain. Many of the constituents are proteolytic enzymes, and with the likelihood that the brain lacks inhibitors normally found in plasma, these enzymes might cause extensive proteolytic cellular damage. The possibility that an inflammatory reaction contributes to the degree of brain damage after acute cerebrovascular lesions is worth further exploration, especially since therapeutic approaches for reducing the inflammatory reaction are available.

Acknowledgment

We thank Mrs. Margit Tjernberg and Ms. Kerstin Lindbladh for their skilful technical assistance. This study was supported by the Swedish Medical Research Council, Gustaf V:e 80 year foundation and the Swedish National Association against Heart and Chest Diseases.

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Lactoferrin, lysozyme, and beta 2-microglobulin in cerebrospinal fluid. Elevated levels in patients with acute cerebrovascular lesions as indices of inflammation.
A Terent, R Hällgren, P Venge and K Bergström

doi: 10.1161/01.STR.12.1.40

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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