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ORIGINAL ARTICLE

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Comparative evaluation of pulmonary lactoferrin and lysozyme immunoreactivity for the postmortem diagnosis of death due to sepsis

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Abstract To determine whether lactoferrin (LF) and lysozyme (LZ) can be used as immunohistochemical postmortem markers of sepsis, pulmonary tissue sections from autopsy cases of sepsis-related fatalities (n=13) and control cases of non-septic fatalities (n=14) were evaluated for differences in leucocytic immunoreactivity. LF and LZ were investigated in paraffin sections using the AEC technique. The immunohistochemical expression of both markers was scored, evaluating the quantity of immunopositive cells and the intensity of the intracellular immunoreactivity. There was a statistically significant association between an enhanced expression of LF on pulmonary leucocytes in sepsis-related fatalities in contrast to non-sepsis cases (P<0.001), whereas no such difference could be observed for LZ immunoreactivity between the two study groups. Pneumonic tissue alterations had no significant influence on LF and LZ immunoreactivity, thus suggesting differences between the degranulation of these non-specific antibacterial agents in local and systemic inflammatory processes. While the variability of LZ immunoreactivity, possibly reflecting a non-specific release from lysosomes according to the length of the postmortem interval, limits its application to the postmortem diagnosis of sepsis, the immunohistochemical detection of an enhanced expression of LF can contribute to the postmortem discrimination between sepsis and non-septic fatalities.

Keywords Lysozyme · Lactoferrin · Pulmonary leucocytes · Neutrophil granules · Sepsis · Lung injury · Postmortem sepsis diagnostics

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Introduction

In sepsis, the organ primarily targeted for injury is the lung, and a progressively impaired lung function is the major complication and cause of death under septic conditions [8, 12, 29, 30]. Thus far, in forensic autopsy practice, the postmortem diagnosis of sepsis is mainly established on macroscopic and routine histological findings that appear predominantly unspecific [21, 23, 25, 26, 28]. However, recent attention has focused on the immunohistochemical detection of an enhanced expression of different markers of the inflammatory cellular response in sepsis-induced lung injury, thus providing relevant information in the forensic postmortem elucidation of death due to sepsis [18, 27]. Accordingly, the present study was performed on human lung tissue originating from sepsis-related fatalities and non-septic fatalities to assess the immunoreactivity of lactoferrin (LF) and lysozyme (LZ), both non-specific antibacterial agents located in granules of leucocytes that modulate the host response to infection and inflammation and to determine whether these markers can contribute to the postmortem diagnosis of sepsis.

Materials and methods

Lung specimens were obtained from four different lung lobes at autopsy from 27 individuals divided in two study groups. The postmortem intervals ranged between 1 day and 3 days.

1. Sepsis group: 13 non-consecutive autopsy cases (seven males, six females; individual ages 27–83 years; mean age 57 years) from the Institute of Pathology, University of Hamburg, Germany, with a diagnosis of sepsis in vivo according to the definition of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [1] and a clinical diagnosis of fatal multiple organ failure due to sepsis. The period of the septic condition ranged between 2 days and

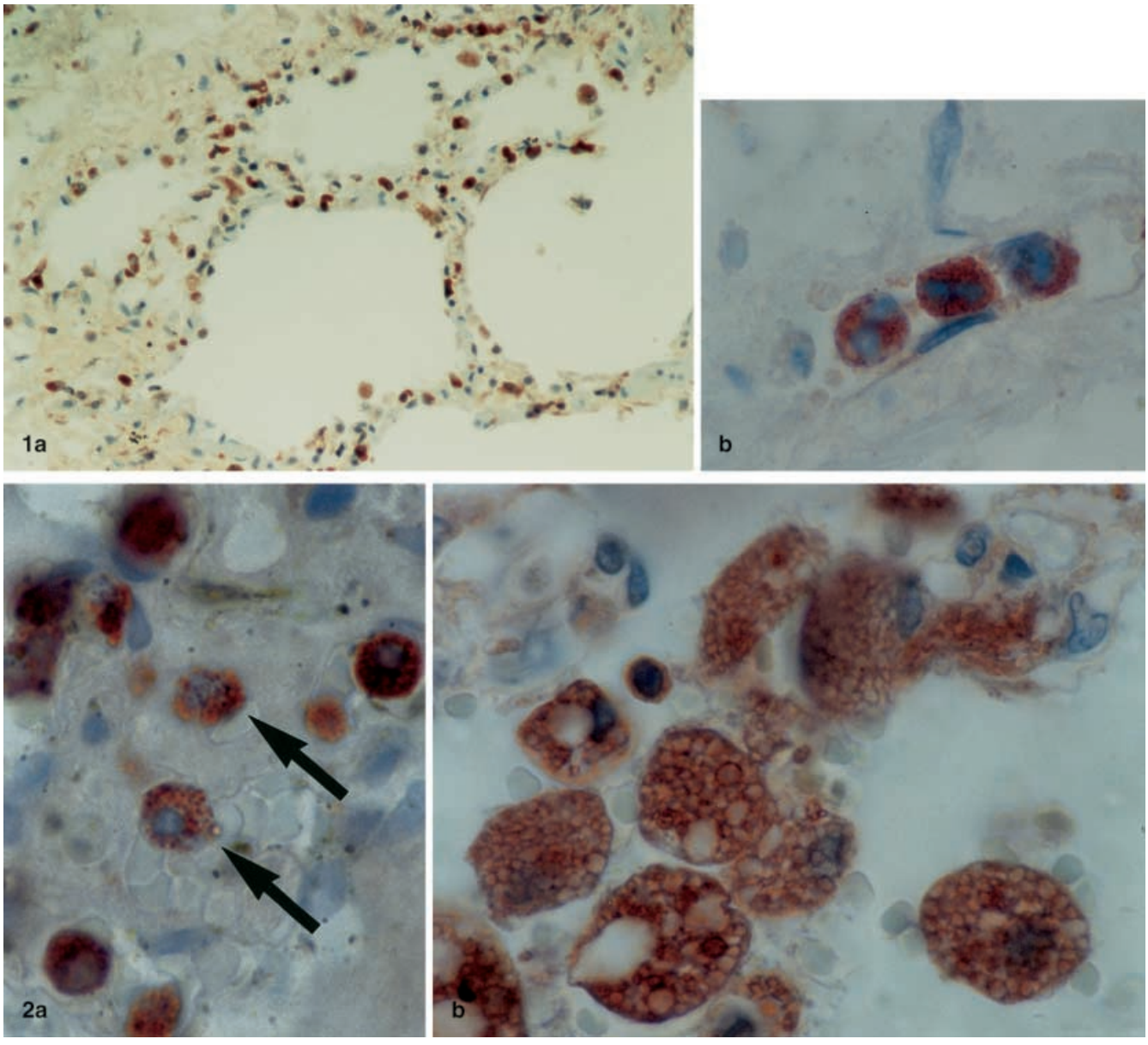

Fig. 1 Lactoferrin (LF)-positive immunoreactivity in sepsis-induced lung injury. **a** Panoramic view of intravascular, interstitial and intra-alveolar leucocytes immunopositive for LF. LF, original magnification ×20. **b** High-power view of intracapillary granulocytes immunopositive for LF expressing a homogenous staining pattern. LF, original magnification ×800

Fig. 2 Lactoferrin (LF)-positive staining reaction in the control group. **a** Granular appearance of intraleucocytic LF deposits (→). LF, original magnification ×800 **b** Vacuolar appearance of LF deposits on macrophages/foam cells. LF, original magnification ×720

33 days; micro-organisms isolated in vivo were *Staphylococcus aureus* (*n*=5), *Pneumocystis carinii* (*n*=2), *Aspergillus fumigatus* (*n*=2), *Klebsiella spec.* (*n*=1), *Pseudomonas aeruginosa* (*n*=1) and *Clostridium difficile* (*n*=1) and cultures were negative in one case. No other cause of death was revealed by postmortem examination and histology.

2. Control subjects: 14 non-consecutive autopsy cases (nine males, five females; individual ages 4–67 years, mean age 48 years) from the Institute of Legal Medicine, Hamburg, Germany, with death due to various natural and unnatural causes (myocardial infarction, *n*=2; carbon monoxide poisoning, *n*=2; electrocution, *n*=2, exsanguation, *n*=2; trauma/polytrauma, *n*=1; pulmonary embolism, *n*=1; intracerebral hemorrhage, *n*=1; hanging, *n*=1; drowning, *n*=1; alcohol intoxication, *n*=1) served as control group. None of the individuals in this study group had a medical history of a septic condition prior to death, but some suffered from a complicating pneumonia after a short survival period following intoxication or trauma.

The lung specimens were fixed in 4% buffered formalin, embedded in paraffin, cut in 4- to 5-µm sections and stained with hematoxylin and eosin and periodic acid–Schiff. In the sepsis group, routine histological examination substantiated the presence of lobar pneumonia in three cases and that of a bronchopneumonia in four cases. In the control group, a lobar pneumonia was found in two cases and a bronchopneumonia was present in two additional cases.

Immunohistochemistry

Samples were fixed in 4% phosphate-buffered saline (PBS) formaldehyde, embedded in paraffin and cut into 6-µm sections. Immunohistochemical stains were carried out with antibodies against LF (Dako, Glostrup, Denmark) and LZ (Dako, Glostrup, Denmark) that were applied using a standard peroxidase-labelled streptavidine-biotin technique, either with microwave heating pretreatment or using conventional methods with trypsination where appropriate. After counterstaining with hemalaun, the sections were finally mounted with Aquatex (Boehringer, Mannheim, Germany). Two negative control sections were used in each case. One was incubated only with the secondary antibody, the other was incubated only with the primary antibody. As a positive control, sections of human submandibular gland were used.

Immunohistochemical grading

The slides were coded and examined without knowledge of their origin from each study group. Ten representative visual fields were randomly selected from the center of the slides and analyzed at 20× magnification. As a semiquantitative measure of the expression of LF and LZ, the leucocytic cell content of immunopositive granules was determined by grading the intracellular staining intensity as (0) no granule staining, (1) weak, (2) moderate and (3) strong, and the total of immunopositive intravascular, interstitial and intra-alveolar leucocytes was classified as (0) no positive cells, (1) <10%, (2) 10–50%, (3) 50–80% and (4) >80% positive cells. After multiplication of both values, the results were graded from 0 (no immunoreactivity in leucocytes) to a maximum score of 12.0 (strong staining intensity in more than 80% positive leucocytes).

Statistical analysis

Statistical analysis of the data was performed using descriptive statistics and an unpaired sample *t*-test and the Mann-Whitney rank sum test with P values <0.01 considered significant.

Results

Expression of LF

In the sepsis group, intravascular, interstitial and intra-alveolar leucocytes and macrophages showed an intense positive staining reaction to LF in all examined lobes of the lungs (mean expression 9.0); whereas, in the control group, LF immunoreactivity was far less intense (mean expression 4.5), displaying a weak to moderate staining for LF in fewer such positive cells. In comparison with the control group, LF expression in the sepsis group differed significantly (P<0.001). While the prevailing number of intravascular, interstitial and intra-alveolar leucocytes and macrophages immunopositive for LF showed a homogeneous staining pattern in the cases making up the sepsis group (Fig. 1), LF deposits had a predominantly granular appearance in leucocytes and a more vacuolar appearance in macrophages/foam cells in the pulmonary tissue sections originating from the non-septic control individuals (Fig. 2). In the sepsis group, leucocytes positive for LF were seen adhering to endothelial cells (Fig. 3). Glandular cells of the bronchi expressed an infrequent weak to moderate immunopositive reaction

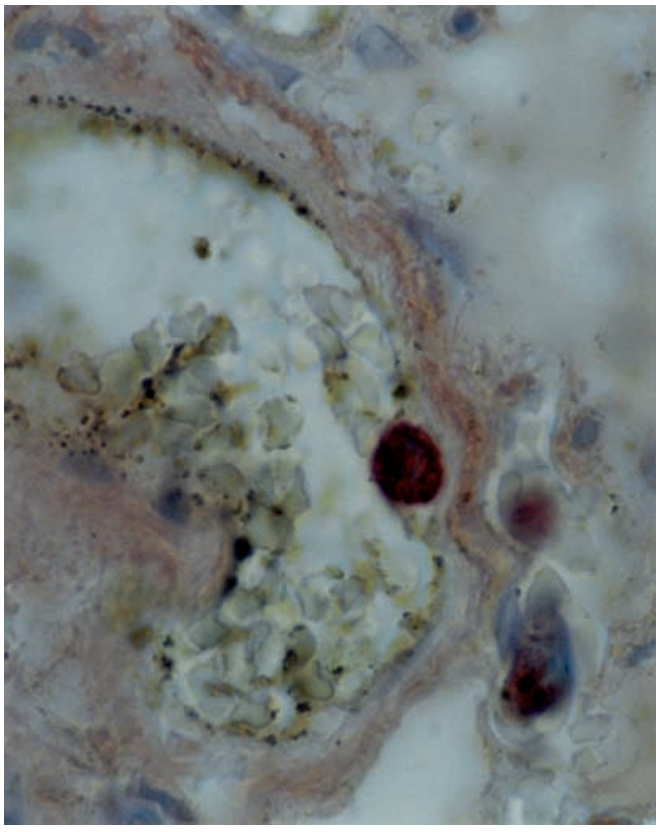

Fig. 3 Sepsis case. High power view of an intravascular leucocyte immunopositive for lactoferrin (LF) adhering to the endothelial cell lining of a pulmonary vessel. LF, original magnification ×800

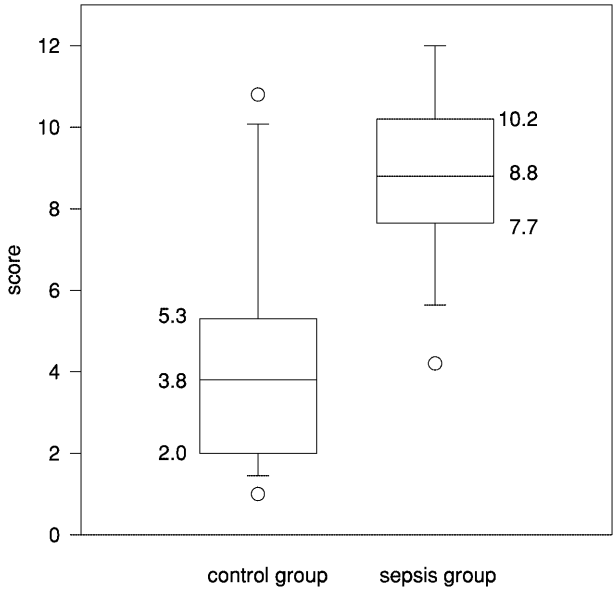

Fig. 4 Immunohistochemical expression of lactoferrin (LF) in the study groups according to the results of the Mann-Whitney rank sum test

Table 1 Results of the semiquantitative grading of the immunohistochemical expression of lactoferrin (LF) using descriptive statistics

Study group	Cases (*n*=27)	Mean expression ±SD	Range	95% Confidence interval
Sepsis group	*n*=13	9.0±2.3	4.2–12.0	7.6–10.4
Control subjects	*n*=14	4.5±3.0	1.0–10.8	2.8–6.2

Table 2 Results of the statistical analysis of the semiquantitative grading of the immunohistochemical expression of lysozyme (LZ) using descriptive statistics

Study group	Cases (*n*=27)	Mean expression ±SD	Range	95% Confidence interval
Sepsis group	*n*=13	8.8±2.8	3.0–12.0	6.6–11
Control subjects	*n*=14	6.8±3.7	2.2–12.0	5.2–8.4

for LF in both study groups. Among the cases in the sepsis group, six had an immunohistochemical grading score of 10.0 or above. Of these, two cases reached the maximum score of 12.0. In contrast, a total grading score of 10.0 or above was evaluated solely in two control subjects, and the maximum score of 12.0 was not reached in any of the cases in the control group (Fig. 4). In the control group, there were no differences between the grade of the intracellular staining intensity and the number of cells positive for LF.

The period of the septic condition and the species of micro-organisms isolated in vivo or a preceding administration of antibiotics had no significant influence on the total score of LF immunoreactivity in the individuals with fatal septic outcome. There were no obvious differences in the quantity of immunopositive cells or in the intensity of intraleucocytic LF immunoreactivity between cases with pneumonic tissue alterations and cases with pulmonary tissue alterations of other causes (e.g., emphysema, shock lung). The results of the semiquantitative grading of the immunohistochemical expression of LF calculated using descriptive statistics are given in Table 1.

Expression of LZ

In both the sepsis group and the control group, a preponderant moderate LZ immunopositivity was observed on a considerable number of such cells in all examined lobes of the lungs (sepsis group: mean expression 8.8, control group: mean expression 6.8; Table 2). There was no statistically significant difference in LZ immunoreactivity referring to the total grading score between the sepsis group and the control group (P<0.152). Intraleucocytic LZ deposits appeared mainly granular in leucocytes and vacuolar in macrophages/foam cells in both study groups. An influence of the length of the preceding sepsis on LZ immunoreactivity or a correlation between pneumonic tissue alterations and the total immunohistochemical grading score of LZ was not detectable. Figure 5 shows the immunohistochemical expression of LZ in both study groups.

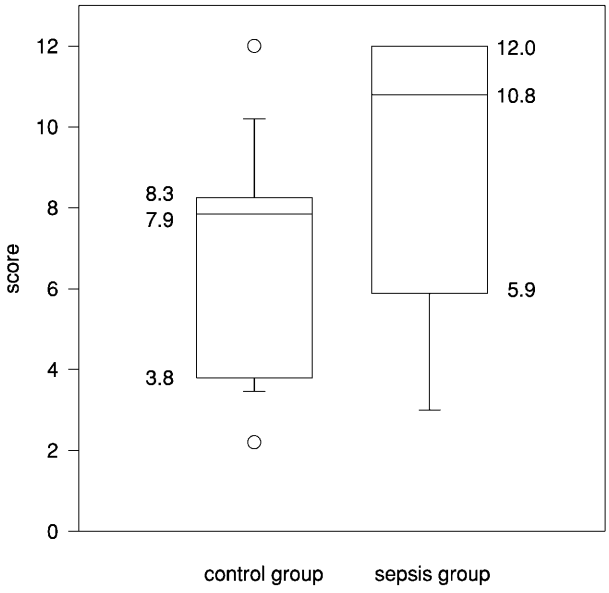

Fig. 5 Immunohistochemical expression of lysozyme (LZ) in the study groups according to the results of the Mann-Whitney rank sum test

Discussion

In the absence of conclusive autopsy and routine histological findings, and when a well-documented anamnesis on the clinical course of a deceased is not available, the postmortem diagnosis of death due to sepsis remains an unsolved problem in forensic casework. LF is an iron-binding glycoprotein located in specific (secondary) granules of leucocytes and plays a central role in the host response to infectious stimuli in providing both bacteriostatic and bactericidal protection [3, 5, 13, 16]. LF's bacteriostatic effect is related to its ability to deprive bacteria of the iron required for growth, and the release of granule lactoferrin promotes the adhesiveness of leucocytes to endothelial surfaces, thus retaining leucocytes at inflammatory sites to amplify the inflammatory response [2, 4, 15, 16, 19].

LZ is a low molecular weight protein, showing bacteriostatic and bactericidal activity. It is mainly found in secondary granules of leucocytes but is also located in primary leucocytic granules (lysosomes) [14]. LZ triggers bacterial autolysis by hydrolyzing the glycoside bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, both components of the peptidoglycan in bacterial cell walls [7, 9, 14, 24].

Elevated plasma levels of LF and LZ have been found in patients with bacteremia and in a variety of inflammatory diseases, and plasma levels of LF are significantly

higher in patients with clinical sepsis when compared with healthy controls [6, 17, 22]. While serum LZ levels correlate with leucocyte death and leucocyte turnover rate [10, 20], serum LF is better correlated with the total leucocyte pool than with the leucocyte turnover rate [11].

The current investigation, assessing the use of an immunohistochemical detection of LF and LZ in human lung autopsy material for the postmortem diagnosis of sepsis, was prompted by earlier studies by Ortmann and Brinkmann (1997) and our investigative group [27], indicating that the immunohistochemical detection of a different pulmonary expression pattern of cellular adhesion molecules, such as P-selectin and E-selectin, can be sufficiently applied to the postmortem differentiation between death due to sepsis and other causes.

In comparing LF and LZ immunoreactivity in sepsis-related fatalities and non-sepsis cases, we found a significant association between an enhanced expression of LF on pulmonary leucocytes and macrophages and a preceding sepsis with fatal outcome, whereas no such association could be observed for LZ immunoreactivity between the two study groups. Referring to the total grading score in the sepsis group, the considerable variability and wide range of LZ immunoreactivity limits the application of this marker to the forensic elucidation of death due to sepsis. The interindividually different leucocytic expression of LZ in both study groups may reflect a non-specific release from lysosomes that is not clear at present. Whether the cause of death or the length of the postmortem interval contributes to this phenomenon cannot be estimated at present due to the limited divergence in the range of the postmortem period (between 1 day and 3 days) of the cases in our study.

Referring to the total of the LF immunohistochemical grading score achieved, there were no obvious differences between cases with pneumonic tissue alterations and cases with pulmonary tissue alterations of other causes in both study groups, suggesting considerable differences between degranulation of LF in local and systemic inflammatory processes. Accordingly, false positive results should not be expected in non-sepsis cases with merely localized pulmonary inflammation. At present, an explanation for the enhanced LF expression in two cases of the control group (both males, 36 years and 40 years, cause of death carbon monoxide poisoning and hanging, respectively) is lacking – autopsy and a thorough histological examination revealed no underlying infectious disease process, especially no (broncho-) pneumonia. The time span of the septic condition and other (individual) clinical and biological parameters had no influence on the total score of LF immunoreactivity.

In conclusion, our results indicate that the immunohistochemical detection of an enhanced expression of LF can give histomorphologic evidence of sepsis-induced lung injury preceding to death. Accordingly, LF can be considered as a valuable immunohistochemical marker for the postmortem discrimination between sepsis and non-septic underlying diseases.

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