Development of a Microparticle-Enhanced Nephelometric Immunoassay for Quantitation of Human Lysozyme in Pleural Effusion and Plasma

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A microparticle-enhanced nephelometric immunoassay, based on polystyrene beads coated with antihuman lysozyme antibody, has been developed for lysozyme quantification in sera and pleural effusions. The standard curve extends from 0.58 mg/l to 18.75 mg/l and no antigen effect was observed. The results showed a good serial precision. The intra-assay precision ($n = 20$) expressed as CV was between 2.2 and 4.2 in three different concentrations. The inter-assay precision, with different calibration curves ($n = 12$) was between 6.4 and 7.1. The analytical assay showed a sufficient linearity ($r > 0.999$). There were no interfer-

ences either with haemoglobin (up to 4 g/l), lipids (up to 0.5%, expressed as 1% Lipofundina® content), or bilirubin (up to 5 mg/dl). The analytical sensitivity was lower than 0.6 mg/l. The correlation with a *Micrococcus lysodeikticus* turbidimetric assay showed a correlation coefficient of 0.915. We have studied 92 patients with pleural effusion. In each case, pleural fluid adenosine deaminase activity and pleural fluid to plasma lysozyme ratio were determined. The lysozyme ratio showed similar clinical sensitivity and specificity as to adenosine deaminase. J. Clin. Lab. Anal. 13:301–307, 1999. © 1999 Wiley-Liss, Inc.

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INTRODUCTION

Human lysozyme (1,4-N-acetylmuramidase, EC 3.2.1.17), a low molecular-weight protein with 123 amino acids (1), is a bacteriolytic enzyme that promotes the dissolution of the procariote cell wall. This ability depends on the catalytic hydrolysis of the β -(1,4)-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine in the polysaccharide component of the bacterial cell wall (2). The presence of lysozyme in the secretions of various serous glands, as well as in polymorphonuclear granulocytes, monocytes, macrophages, and in Kupffer cells of liver is in accordance with the widely held view suggesting that lysozyme participates in the nonspecific defense system of humans. The enzyme, mainly degraded in the kidney, is believed to be filtered through the glomeruli and resorbed in the proximal tubules where it is catabolyzed (3).

Lysozyme is present in many body fluids such as serum, pleural fluid, cerebrospinal fluid, and amniotic fluid. Because of this wide distribution, lysozyme has been measured to characterise and diagnose a variety of diseases. Thus, there are many reports of changes in serum and urinary lysozyme activity in various kidney diseases, after bilateral nephrectomy, in chronic hemodialysis patients, and in transplantation (3). In haematology, increased levels of the enzyme have

been described in serum and urine of patients with monocytic and myelomonocytic leukemia (4).

Lysozyme quantification in cerebrospinal fluid (CSF) has been shown to be useful in distinguishing viral from bacterial meningitis. A large increase of lysozyme in CSF has also been described in patients with tuberculous meningitis, neurosyphilis, and fungal meningitis (1). Increased serum lysozyme activity has been reported in pulmonary tuberculosis (5,6,7) sarcoidosis (8) and Crohn's disease (4,8). The pleural tuberculous diagnosis relies on pleural biopsy and bacterial culture of pleural fluid, yet problems are frequent. Many reports have demonstrated that determinations of pleural adenosine deaminase (ADA) and the pleural lysozyme to plasma lysozyme ratio are useful in the differential diagnosis of tuberculous and nontuberculous pleural effusions (9,11).

Lysozyme has been measured by different methods. Traditionally, most of the assays have been based on a clearing phenomenon involving a sensitive organism, *Micrococcus lysodeikticus*. So, the quantifying of the enzyme activity was

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commonly measured in human body fluids by turbidimetric (10–13), nephelometric (2,14), or the commercial Lysoplate™ techniques (5,8,9,15). The methods differ extensively with respect to some factors that affect lysozyme activity—e.g., buffer composition, pH, ionic strength, concentration of the *Micrococcus Lysodeikticus* substrate, temperature, and duration of incubation, as well as differences in the source and preparation of the enzyme calibrators (13). All of these factors have led to differences in the reported normal values for lysozyme and have thus limited the clinical utility of the assays. Other procedures described for lysozyme quantitation are immunohistochemistry (16), luminiscence immunoassay (17), and radioimmunoassay (18,19).

Microparticle-enhanced nephelometric immunoassay has been found as a robust analytical technology (20). Improvements in the production of antisera and particles have enabled the development of a wide number of protein assays into the routine laboratory by means of this technology. In addition, these homogeneous assays, automated in new-generation nephelometers, are fast and have all their operations automatically performed, including standard curve preparation, dilution of samples and controls, and results calculations.

Here, we present the development of a microparticle-enhanced nephelometric immunoassay intended for the measurement of lysozyme in pleural fluid and plasma samples.

The aim of this work is an analytical evaluation of the method and to study the possible use of this technology for routine practice.

MATERIALS AND METHODS

Samples

Paired heparin-anticoagulated plasma and pleural fluids were obtained from patients pertaining to the different clinical departments of our hospital, presenting with an undiagnosed pleural effusion. The samples that were not measured immediately were stored in plastic containers at -50°C until analysis. Only one freeze/thaw cycle was carried out.

Apparatus

We used a Dade-Behring BNII nephelometer analyzer (Dade-Behring, Marburg, Germany), which is a fully automated immunochemical analysing system equipped with a fixed-time assay device. The light source is a diode with a wavelength of 840 nm and the signal is measured at an angle of $12\text{--}24^{\circ}$.

Reagents

Reagent 1

Carboxylated polystyrene latex particles with a diameter of 150 nm (Polymer Laboratories LTD., Church Sireton, England), were covalently coated with antihuman lysozyme

antiserum (Ig fraction of rabbit antihuman lysozyme, reference number A 0099, Dako Glostrup, Denmark). The preparation of conjugate was made following a previously described protocol (21). A 1/10 ratio of purified immunoglobulin/microparticles suspension was used for the formation of imine bonds between the carboxylic latex group and the ϵ -amino groups in the antiserum. The purified antiserum used was specific for human lysozyme; traces of contaminating antibodies had been removed by the manufacturer using solid-phase absorption with human plasma and urine proteins. The specific reaction against human lysozyme was ascertained using crossed immunoelectrophoresis, which revealed that the lysozyme precipitation arc appears only against concentrated urine from patients with monocytic leukemia. No precipitation was observed against nondiseased human plasma or concentrated nondiseased human urine.

Reagent 2

An *N*-Reaction buffer (Dade-Behring) was used: phosphate 50 mmol/l with NaCl 0.2 mol/l and polyethylene glycol (PEG), that was used as a reaction enhancer.

Dilution Buffer

An *N*-diluent (Dade-Behring) phosphate buffer was used for the automatic dilution of standard, control and patients samples.

Standard

Chromatographical purified human milk lysozyme was purchased from Sigma Chemical Co., St Louis (product number L-6394). This material was diluted in phosphate buffer 66 mmol/l pH 7.5, containing 31 mmol/l of NaCl, 18 mmol/l of sodium azide per liter, and 2 g/l of bovine serum albumin. This calibrator had a lysozyme concentration of 15 mg/l.

Principle

Polystyrene particles covalently coated with antilysozyme antibodies are agglutinated by the lysozyme contained in the samples, thereby forming larger particles, which may scatter the light. The scattered-light signal of the reaction solution is measured at 10 sec and 12 min. The difference between the two signals is used to calculate the lysozyme concentration of the samples. Results are evaluated by the instrument using a log-logit function and are expressed in mg/l.

Assay Procedure

The microparticle-enhanced nephelometric immunoassay of lysozyme was performed automatically by the instrument. In essence, 60 μl of lysozyme standard (twofold diluted from 1:40 to 1:1280), control, or unknown samples (1:100 dilu-

tions) are added along with 20 μ l of antibody-coated beads, 30 μ l of *N*-Reaction buffer, and 120 μ l of *N*-diluent into a reaction cuvette; this mixture is incubated at room temperature for 12 min. When calibrated, the instrument builds a six-point curve with the increments of nephelometric units; these results are stored for subsequent recall and comparison with the nephelometric units readings of the samples. Samples of high concentration are automatically remeasured at the next highest dilution (1/400, 1/2000), extending the assay range up to 360 mg/L of lysozyme.

Comparison Study

Correlation was carried out on 25 plasma samples by means of a *Micrococcus Lysoideikticus* turbidimetric assay. Dr. Torrents (Reference Laboratory, Barcelona, Spain) kindly provided these measured samples.

Interfering Substances

Lipofundina® and bilirubin were purchased from Brana (Barcelona, Spain) and Sigma Chemical Co.(St Louis, MO), respectively.

Clinical Studies

There are many reports that have shown the importance of the determination of pleural ADA and the pleural fluid to plasma lysozyme ratio in the differential diagnosis between tuberculous and nontuberculous pleural effusions (4,5,6,9,11).

We have studied 92 patients with pleural effusion. In each case, pleural fluid ADA and pleural fluid to plasma lysozyme ratio were calculated. The patients were classified into the following groups:

- Six tuberculous effusions were found. The diagnosis was determined by culture of fluid or pleural biopsy or by the presence in the pleural biopsy of granulomas with necrosis and compatible clinical and therapeutic evolution.
- Malignant effusions occurred in 23 patients. The diagnosis was obtained by pleural biopsy with typical histopathologic or cytological findings with compatible clinical findings and evolution.
- Transudates were found in 31 patients. All of them had cardiac failure. This diagnosis was made on the basis of clinical and radiologic findings, the disappearance of the effusion on suitable diuretic treatment, and the absence of pulmonary infiltrates or malignancy.
- Empyemas were found in nine cases. The diagnosis was determined in accordance with its definition as parapneumonic effusions with positive culture results.
- Parapneumonic effusions occurred in 23 patients. The effusion was associated with a bacterial pneumonia on the basis of compatible clinical and radiologic findings.

Statistical Analysis

Statistical significance of differences between mean values was tested by one-way analysis of variance (ANOVA). Curves of sensitivity vs. specificity were used to establish threshold values for each parameter (Receiver operating characteristic, ROC).

RESULTS

Calibration Curve

The reproducibility of the calibration curves was estimated by measuring the light scattered by each dilution of lysozyme standard on 12 different days. The resulting calibration curve (Fig. 1) shows a typical sigmoidal shape as in other immunoassays. The range extends from 0.58 mg/l to 18.75 mg/l. The calibrations were stable for at least 15 days.

Precision

Measuring lysozyme levels in four human sera pools assessed the precision of the immunoassay. The intra-assay precision was measured with 20 replicates at three lysozyme values ranging from 2.9 mg/l to 15.2 mg/l. For the interassay precision ($n = 12$) twelve different calibration curves were used. Data are given in Table 1.

Linearity, Sensitivity, and Prozone Phenomenon Studies

Linearity of four different samples, containing lysozyme values within the range of analysis, was evaluated using serial dilutions prepared with saline. The results are shown in Figure 2. The deviation of the measures from the theoretic values did not exceed the 5% level.

The detection limit of the assay, obtained with ten replicates of a saline solution was less than the end of the calibra-

Fig. 1. Calibration curve of the lysozyme assay.

TABLE 1. Intra- and interassay precision

Intra-assay (n = 20)	Pool I	Pool II	Pool III
x, mg/l	15.18	7.20	2.90
S.D., mg/l	0.64	0.32	0.06
C.V., %	4.2	4.5	2.2
Interassay (n = 12)	Pool IV	Pool II	Pool III
x, mg/l	22.30	6.66	2.95
S.D., mg/l	1.43	0.47	0.20
C.V., %	6.4	7.1	7.0

tion curve (0.58 mg/l). The assay does not show prozone phenomenon up to 180 mg/l (Fig. 3).

Interference Studies

For the interference studies we followed the procedure of Glick (22). We assessed the effect of bilirubin and haemoglobin by adding known amounts of these substances to a baseline serum pool containing 3.0 mg/l of lysozyme. Interference from lipemia was assessed with increasing quantities of Lipofundina®. No effects were seen in any sample with levels up to 1% of Lipofundina® (113 mmol of tryolein), 20 mg/l of bilirubin, or 4 g/l of haemoglobin (Fig. 4).

Comparison Study

Correlation between the nephelometric latex immunoassay and the turbidimetric *M. lysodeikticus* method is shown in Figure 5. The regression equation was $y (\text{neph}) = 0.77x (\text{turb}) - 0.62$, and the correlation coefficient was 0.915, indicating a close correlation between the two assays.

Clinical Studies

The results of the pleural fluid/plasma lysozyme ratio and pleural fluid ADA obtained in the different pleural effusions

are shown in the Figure 6. In the tuberculous group, the pleural fluid/plasma lysozyme ratio and pleural adenosine deaminase activity were significantly higher ($P < 0.01$) than in malignant effusions, transudates, and parapneumonic groups. Table 2 shows the sensitivities and specificities for the pleural fluid/plasma lysozyme ratio and adenosine deaminase activity in tuberculous and nontuberculous pleural effusions. Our PPV and NPV, including empyemas, are 30 and 98% for lysozyme, respectively, and 26 and 97% for ADA.

DISCUSSION

Elevated lysozyme levels in biological fluids have been reported in many diseases, e.g., monocytic and myelomonocytic leukemias, renal dysfunctions, granulomatosis diseases such as sarcoidosis, Crohn's disease, leprosy, and pulmonary tuberculosis. Nevertheless, most of the actual analytical methods for lysozyme determination present several limitations such as poor detection limit, the need of sample pretreatment, unknown interferent factors in biological fluids altering the enzymatic activity of lysozyme, long incubation periods, the constraints due to washing and phase separation or the use of radioisotopes (14).

Our results indicate that the present nephelometric latex immunoassay for lysozyme is easy to perform, rapid (12 min), and allows specific lysozyme measurement in pleural fluids and plasma. The concentration of lysozyme is measured over a large range of concentration with high reproducibility (interassay and intra-assay CVs lower than 7.1%). Prozone phenomenon did not show up to 180 mg/l. The assay was unaffected by any of the added interferents—triglycerides (in the form of a lipid emulsion), bilirubin, or haemoglobin—at concentrations higher than those expected in a routine clinical laboratory setting.

The covalent coupling of proteins such as IgG to latex particles has been shown to present several advantages over

Fig. 2. Assay linearity of the nephelometric assay.

Fig. 3. Antigen excess measurement for lysozyme assay.

simple adsorption procedures, such as the improvement in sensitivity and a superior stability of the reagents (23). This is reflected in our assay by the good reagent and calibration stabilities, where acceptable performance is observed for up to 15 days, allowing for well-spaced calibrations.

We have observed different reference values reported in most published works. These findings could be explained by several lysozyme sources used to prepare the calibrator (hen egg white and man) (24). Gorin et al. showed that different commercial preparations of *Micrococcus lysoideikticus* differ in their susceptibility to lysozyme (13). Another explanation is that, either in turbidimetric method or in the nephelometric method, *Micrococcus lysoideikticus* are lysed

rapidly in suspension, whereas in the Lysoplate™ method *Micrococcus lysoideikticus* are lysed during a diffusion process in agarose with a lengthy incubation. Moreover, in this last procedure, the zones of lysis are difficult to measure accurately. Using a latex particle-enhanced technology we have solved the problems around measuring the lytic activity of lysozyme and the differences between the commercial preparations of *M. lysoideikticus*. Furthermore the presence of possible lysozyme inhibitors (e.g., circulating glycosaminoglycans) that could cause charge interactions with cationic lysozyme and subsequent interference, particularly in the Lysoplate™ assay (24), have been avoided with the use of specific antibodies.

Fig. 4. Interference studies of the nephelometric assay.

Fig. 5. Correlation between lysozyme measured by the nephelometric latex immunoassay and the turbidimetric *M. liseideikticus* method.

The presence of a raised pleural fluid/plasma lysozyme ratio suggests a local production of lysozyme in the pleural cavity, such as would occur in tuberculous pleuritis and empyemas (10). This synthesis has been demonstrated in epithelioid cells and mononuclear phagocytes of tuberculous granulomas, as well as in granulocytes of empyemas (25). Empyemas do not cause difficulty in diagnosis because of their fluid appearance and biochemical and bacteriologic features (9). In this kind of pleural fluid the values of lysozyme and ADA are similar to those of the tuberculous group. The opposite situation is found in neoplastic effusions. In these effusions, just as in the group with transudates, the existence of a low pleural fluid/plasma

lysozyme ratio would suggest a predominance of lysozyme diffusion from the systemic circulation (10).

Tuberculous pleurisy has an important place in the differential diagnosis of pleural effusions, particularly in areas where tuberculosis is still prevalent. Diagnosis is often difficult to establish, especially in cases where cultures for the isolation of *M. tuberculosis* and closed pleural biopsy are inconclusive; thus, the availability of a test that could yield useful information in the differential diagnosis is needed. Lysozyme quantification, as shown by the sensitivity and specificity obtained in our patients, seems to be useful as an initial clinical orientation in the study of pleural effusions with a diagnostic efficiency similar to adenosine deaminase determination. Both parameters showed similar sensitivities, although lysozyme specificity was lower than that of ADA. Nevertheless, the latex-particle technology has the advantage of being a rapid method: full automation of the procedure in a routine nephelometer.

In summary, the use of a latex particle-enhanced technology in order to measure lysozyme simplifies the diagnosis of a wide number of disorders by means of making the laboratory procedure practical for routine practice.

Fig. 6. Pleural fluid to plasma lysozyme ratio and adenosine deaminase in patients with effusion of various causes.

TABLE 2. Sensitivity and specificity in tuberculous vs. nontuberculous effusions

	Sensitivity(%)	Specificity (%)
ADA ^a	83.3	87
Lysozyme ^b	83.3	70

^aCut-off = 40 U/L.

^bCut-off = ratio > 2.

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