Radioimmunoassay for Urinary Lysozyme in Human Serum from Leukemic Patients

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We present a radioimmunoassay for lysozyme in human serum, based upon human lysozyme isolated from the urine of leukemic patients and antisera prepared against this lysozyme in the goat. In the separation step, a second antibody is used. By properly adjusting the concentrations of unlabeled and \(^{125}\)I-labeled lysozyme and of the antibodies, maximal precision (SD, 0.04 mg/litre) was obtained in the range 0.00 to 2.00 mg/litre. In 20 normal volunteers the lysozyme concentration was 4.6 ± 0.8 mg/litre (mean ± SD), in 13 patients with monocytic leukemia 34.4 ± 8.6 mg/litre. Correlation with lysoplate determinations was excellent in leukemic sera (r = 0.97) but was poor in normal sera (r = 0.35), possibly owing to the existence of isoenzymes.

Additional Keyphrases: normal values • lysoplate assay compared • possible lysozyme isoenzymes • cancer

Although lysozyme (EC 3.2.1.17) can be assayed by several methods, none of them may be considered as satisfactory for analytical purposes (1). The problems connected with lysozyme assays have recently been illustrated by the discussion concerning the diagnostic value of serum lysozyme determinations in gastrointestinal diseases (2, 3), where differences in methodology were evoked as the cause of the observed discrepancies (4–6). The use of different standards and conventions in converting or expressing lysozyme activities into quantities has added to the confusion. In view of these problems, we decided to develop a radioimmunoassay for human lysozyme.

Materials and Methods

Reagents

Lysozyme was isolated from the urine of a patient with monocytic leukemia, by the method of Canfield et al. (7), with minor modifications. The purity of the isolated product was established by electrophoresis on cellulose acetate and on polyacrylamide, by isoelectric focusing, and by gel filtration. Concentrations in standards were calculated by measuring the absorbance of stock solutions containing about 0.1 g/litre and assuming an absorbivity of 2.55 at neutral pH for a concentration of 1 g/litre (7). Carrier-free Na\(^{125}\)I and \(^{22}\)NaCl was obtained from IRE, Fleurus (Belgium), lactoperoxidase (EC 1.11.1.7) and hen's egg-white lysozyme from Sigma Chemical Co., St. Louis, Mo. 63178. The goat antihuman lysozyme and the rabbit antigoat immunoglobulin antisera were from Nordic Pharmaceuticals and Diagnostics, Berchem, Belgium.

Lysoplate Assay

For lysoplate determinations (6) we used gels (10 g of agarose per litre) containing per litre, 0.6 g of Micrococcus lyso-deikticus and 66 mmol of phosphate, pH 6.6. We used a sample volume of 10 μl.

Iodination of Human Lysozyme

Iodination was done by the lactoperoxidase method (8). The reaction mixture contained 0.2 mCi of \(^{125}\)I (10 μl), 80 μg of lysozyme (100 μl), and 0.5 μg of lactoperoxidase (10 μl). The reaction was started by adding 10 μl of 0.86 mmol/litre H\(_2\)O\(_2\) and terminated after 15 min by adding 100 μl of sodium metabisulphite (0.5 g/litre) and 200 μl of potassium iodide (31.1 g/litre). The reaction mixture was applied to a Sephadex G-10 column (total volume, 13.0 ml) equilibrated with diethyl barbiturate HCl (20 mmol/litre, pH 7.2) containing 10 g of bovine serum albumin per litre. One-millilitre fractions were collected and the radioactivity in 1-μl aliquots was counted.

Radioimmunoassay

To set up the radioimmunoassay, we adjusted the specific activity of the labeled lysozyme to 0.14 μCi/ng by adding unlabeled enzyme. In assay mixtures a total of 10 000 cpm \(^{125}\)I was used, corresponding to 50 ng of human lysozyme. A dilution curve of the antiserum was prepared according to usual procedures. A binding of 53% was observed with a 200-fold dilution, which was therefore selected as the working dilution.

The radioimmunoassay mixture always consisted of 100 μl of goat antihuman lysozyme (200-fold dilution), 100 μl of either sample, standard or buffer, 100 μl of iodinated lysozyme (50 ng), and 100 μl of assay buffer.

The assay buffer, which also was used for all necessary dilutions, was barbiturate-HCl (20 mmol/litre, pH 7.2) containing, per litre, 40 g of bovine serum albumin and 0.1 mCi of \(^{22}\)Na. After a 24-h incubation at 4°C, 50 μl of goat γ-globulins (suitably diluted) was added, followed by 50 μl of rabbit antigoat γ-globulins. This mixture was incubated for an additional 2 h at 25°C, and, after 800 μl of cold NaCl solution (9 g/litre) was added, was centrifuged at 2000 × g for 30 min. Supernate and precipitate were separated by aspiration and both were counted for \(^{22}\)Na and \(^{125}\)I in a gamma ray spectrometer. \(^{125}\)I was counted from 10 to 70 keV, \(^{22}\)Na from 470

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to 550 keV. The spill-over of $^{22}$Na counts in the $^{125}$I channel was determined in every assay, and amounted to approximately 0.14. $^{125}$I counts were corrected accordingly and $^{125}$I counts in precipitate and supernatant were calculated, assuming a uniform distribution of $^{22}$Na in the soluble phase. The standard curve was prepared by adding 0.0025, 0.05, 0.1, 0.2, 0.4, and 0.8 $\mu$g of human lysozyme in 100 $\mu$l of buffer containing bovine serum albumin (40 g/litre). Unknowns were routinely diluted threefold in buffer containing albumin. Sera from leukemia patients were diluted, based upon results of their lysoplate determinations, so as to fall within roughly the same assay range. The amount of carrier goat $\gamma$-globulins added in the separation phase was separately determined in every experiment. To different tubes, containing the mixture for which maximal complex formation was expected, various amounts of carrier goat $\gamma$-globulins were added, followed by 50 $\mu$l of rabbit anti-goat $\gamma$-globulins. The amount giving maximal precipitation was then used for the assay. The procedure is summarized in Figure 1.

**Results**

Separation of the labeling mixture on Sephadex G-10 is shown in Figure 2. The first peak, corresponding to lysozyme, contained 49% of the total eluted radioactivity. These fractions were combined, and from a determination of the lysozyme concentration by the lysoplate method the specific activity was computed to be 0.6 Ci/g. The specific activity was decreased to 0.14 Ci/g by addition of nonlabeled enzyme. Total lysozyme concentration was 0.5 mg/litre, and 100 $\mu$l of this solution was used in the radioimmunoassays.

A typical standard curve is shown in Figure 3. Precision was computed from the differences between duplicates. For the standard curve the standard error of the experimental parameter (% precipitated) was 0.8%. In the linear part of the curve a 10% decrease in the percent precipitated corresponded to the addition of 50 ng of lysozyme, so that the standard error for the lysozyme determinations may be set at 4 ng. As can be seen from Figure 3, the zone of maximum precision (the linear portion of the standard curve) extended from 0–2 mg/litre (i.e., 0–200 ng added in the test). For all values reported in this paper, sera were suitably diluted to fall within this range. Lysozyme was determined in 13 sera of patients with monocytic leukemia and in 20 normal volunteers, and the radioimmunoassay values were compared with lysoplate determinations. Mean values and standard deviations in the two sets of samples were $34.4 \pm 8.6$ and $4.6 \pm 0.8$ mg/litre by radioimmunoassay, as compared with $30.4 \pm 20.1$ and $5.2 \pm 1.9$ mg/litre by the lysoplate method. Correlation for the two methods was excellent in the leukemia patients ($r = 0.97$) but poor in normals ($r = 0.29$, Figure 4). Cross-reactivity with hen’s egg-white lysozyme was virtually absent (Figure 3).

**Discussion**

The most surprising and at first sight probably disappointing feature of our radioimmunoassay for human lysozyme is its low sensitivity. There are several reasons for this.

When we attempted to iodinate lysozyme at high specific activity, the product was immunologically unreactive. With the Chloramine T method we never obtained a satisfactory product. With the lactoperoxidase method described in this
paper a specific activity of 9 kCi/mol is achieved, but if all six

antibodies of human lysozyme were moniodinated the specific activity could be 12.6 MCI/mol.

However, to measure lysozyme in biological fluids such high sensitivity is not required and might even be a disadvantage. With our labeling procedure, and assuming a total of 10 000 cpm in the assay mixture, the amount of labeled product used in the assay would be about 10 ng, and corresponding amounts could be measured. In serum the normal range is 3.0 to 6.2 mg/litre, or 300 to 620 ng/100 µl, and a dilution of at least 20-fold would be required before the assay. A compromise was sought by decreasing the specific activity to 0.14 Ci/g. With 10 000 cpm in the assay, the zero standard contains 50 ng. The resulting standard curve had highest sensitivity in the 0–2 mg/litre range (0–200 ng in the assay), and even this requires a threefold dilution of most serum samples.

Another unusual feature of our assay system is the high albumin concentration. Initially no albumin was added. In comparison with the present procedure, the percentage of label precipitated by the second antibody was then 3–5% greater in all standards, and in a blank prepared without antibody, nonspecific precipitation was higher than the zero standard! Apparently, lysozyme was denatured during incubation, but was partly protected against denaturation by antibody. Addition of bovine serum albumin at a concentration of 4% protected the blanks, standards, and samples from denaturation (nonspecific precipitation lower than 5%).

The correlation studies with lysozyme determinations raise an important point. Although the concentration range in leukemia patients and normals is quite different, a different precision of the assays at different concentrations cannot be responsible for the low correlation in normal individuals, because leukemia sera were first diluted to the normal range and then tested by both lysozyme and radioimmunoassay. Our findings could be explained by the existence of lysozyme iso-enzymes. In patients with monocytic leukemia the high lysozyme concentration is certainly of monocytic origin and is correctly measured by a radioimmunoassay based upon such lysozyme, but in normal people other sources could contribute to the serum lysozyme. Because antibodies against monocytic lysozyme have been used to localize lysozyme in several tissues (9, 12) lysozymes from different tissues show at least cross-reactivity with monocytic lysozyme. However, this does not exclude the possibility that on a quantitative basis their reactivity in radioimmunoassay could be less.

Recently, evidence has been presented for the existence of lysozyme isoenzymes in the urine of patients with monocytic leukemia (13). We have already suggested that the poor correlation between turbidimetric and lysozyme determinations could be due to the presence of isoenzymes, because in these methods a different criterion determines the final result: in the turbidimetric determination lytic activity is measured, the lysozyme method measures the diffusion zone produced by lysis (5). Such an hypothesis is difficult to substantiate by these methods, because it currently is impossible separately to measure lytic activity or diffusion from the supposed isoenzymes. Similarly, owing to the cross reactivities to be expected between lysozyme isoenzymes in man, a discrimination by radioimmunoassay might prove to be difficult. However, in view of the possible diagnostic implications, efforts should be continued in this direction.

Our radioimmunoassay is a clear improvement over methods based upon activity measurements such as the turbidimetric method and the lysozyme method, which are so easily influenced by assay conditions (14). Also, no other immunologic method matches the precision and accuracy of radioimmunoassay. However, as in any immunologic phenomenon, only the antigen corresponding to the antibody used is detected.

References