Measurement of Urinary Lactoferrin as a Marker of Urinary Tract Infection

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The usefulness of the measurement of urinary lactoferrin (LF) released from polymorphonuclear leukocytes and of an immunochromatography test strip devised for measuring urinary LF for the simple and rapid diagnosis of urinary tract infections (UTI) was evaluated. Urine specimens were collected from apparently healthy persons and patients diagnosed as suffering from UTI. In the preliminary study, the LF concentrations in 121 normal specimens and 88 specimens from patients (60 with UTI) were quantified by an enzyme-linked immunosorbent assay. The LF concentration was 3.300.0 ± 64.3 ng/ml (average ± standard error of the mean) in the specimens from UTI patients, whereas it was 30.4 ± 2.7 ng/ml and 60.3 ± 14.9 ng/ml in the specimens from healthy persons and the patients without UTI, respectively. Based on these results, a 200-ng/ml LF concentration was chosen as the cutoff value for negativity. Each urine specimen was reexamined with the newly devised immunochromatography (IC) test strip to calculate the indices of efficacy. Based on the cutoff value, it was calculated that the sensitivity, specificity, and positive and negative predictive values of the IC test were 93.3, 89.3, 86.2, and 94.9%, respectively, compared with the results of the microscopic examination of the urine specimens for the presence of leukocytes. The respective indices for UTI were calculated as 95.0, 92.9, 89.7, and 96.6%. The tests were completed within 10 min. These results indicated that urine LF measurement with the IC test strip provides a useful tool for the simple and rapid diagnosis of UTI.

Urinary tract infections (UTI) are among the most common diseases of humans, and large numbers of urine specimens are processed daily in most clinical microbiology laboratories. The screening of these specimens for microorganisms is a repetitive procedure with a quantitative distinction between positive and negative results (9, 23). The majority of the test results are negative (11, 14), leaving positive specimens for further processing. Most such tests are relatively simple and reliable but require an overnight incubation of cultures before results are available. Therefore, a rapid screening test would be advantageous if it greatly reduced the time spent on specimens which prove to be negative (thereby allowing the majority of negative reports to be issued the same day that the specimens are received) and required the culture of only positive specimens. The detection of pyuria, in which polymorphonuclear leukocytes (PMNs) are present due to inflammation of the UT, may aid in the diagnosis of UTI (1, 16); pyuria accompanied by a low or negligible bacterial count is a significant finding (13, 15, 20, 25, 26). The presence of asymptomatic and bacteriuric pyuria may indicate infection rather than colonization (10, 17, 27). However, the current methods for detecting pyuria require some experience on the part of the microscopist and can also be time-consuming. A rapid report of results leading to a diagnosis of UTI would be useful to the physician and patients because unnecessary therapy might be avoided.

A test using the esterase activity of PMNs combined with the azo-coupling reaction (5, 19, 24) was reported to simplify the diagnosis of UTI. In addition to esterase, PMNs contain another component, the stable iron-binding protein lactoferrin (LF), in their nuclei and secondary granules (6, 21). The present study was carried out to test the usefulness of the measurement of urinary LF for the diagnosis of UTI and to evaluate the immunochromatography (IC) test strip devised for measuring urinary LF.

MATERIALS AND METHODS

Urine specimens and UTI criteria. The first-voided morning urine specimens were obtained from apparently healthy persons (72 men aged 24 to 59 and 49 women aged 20 to 55). Urine specimens of outpatients were also collected from 45 men aged 40 to 83 (catheterized, 20%; midstream, 27%; and unspecified, 53%) and from 43 women aged 2 to 87 (catheterized, 42%; midstream, 2%; and unspecified, 56%). The specimens were stored immediately at 4°C and submitted to the clinical laboratory within 2 h. The calibrated-loop technique for bacterial culture and microscopic examination of uncentrifuged urine specimens were carried out according to the method of Claridge et al. (2).

The guidelines to establish a test result as UTI positive for the purpose of this study were as follows: (i) for acute cystitis, which is one of the uncomplicated UTIs, female population aged 16 to 69, dysuria, frequency, urgency, and/or suprapubic pain, PMNs at ≥10/mm³, and a bacterial count of ≥10⁵ CFU/ml of at least one clearly predominating organism before antibiotic therapy; (ii) for acute pyelonephritis, which is also one of the uncomplicated UTIs, female population aged 16 to 69, fever of ≥37°C with flank pain, PMNs at ≥10³/mm³, and a bacterial count of ≥10⁴ CFU/ml; (iii) for complicated UTI; both male and female populations having at least one underlying disease of the UT, PMNs at ≥10³/mm³, and a bacterial count of ≥10⁴ CFU/ml.

Preparation of PMN lysate. PMNs were collected from normal heparinized venous blood according to the method of Ferrante and Thong (4). The PMNs were suspended in 100 mM borate-buffered saline (BBS), pH 8.2, containing 0.07% Zwittergent 3-14 (Calbiochem-Novabiochem, La Jolla, Calif.), 0.07% CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate) (Dojindo, Kumamoto, Japan), 0.07% BIGCHAP (Dojindo), 0.2% Triton X-100 (Bio-Rad, Hercules, Calif.), and 0.5% bovine serum albumin (BSA; Sigma, St. Louis, Mo.)
phenylphosphate solution (1 mg/ml in 9.7% diethanolamine and 0.01% MgCl₂) to serve as a sink for any excess volume of liquid sample. Then another absorbent pad made from cellulose (5 by 20 mm) was incorporated to the support at the upstream end with 2 mm overlapping with the membrane. A polystyrene support (5 by 80 mm) was laminated on the back of the antibody solution (240 μg/ml) was added and allowed to react for 1 h at 5°C for 30 min. The final pellet was resuspended in the same buffer, and the absorbance of each well was stopped by the addition of 4 M NaOH solution, the absorbance of each well was measured with a microplate reader (EL 312e; Bio-Tek Instruments, Winooski, Vt.).

IC of LF. All solutions and buffers used to prepare the IC test strip were filtered with 0.22-μm-pore-size membrane filters before use. 17B04-08 antibody (3 mg/ml in BBS) was deposited on an Immunodyne ABC membrane (3-μm pore size) (Pall Gelman Sciences, New York, N.Y.), which is made from nylon66 and has a high density of chemically activated covalent binding sites against amino groups, and 32D01-10 [lgG2a(ε)] was used as the antibody conjugated with AuroBeads G10 colloid gold particles (Amersham) for the sandwich-type IC system.

Sandwich ELISA of LF. The polyclonal antibody to LF (10 μg/ml) was immobilized in each well of 96-well microtiter plates (catalog no. 3590; Costar, Cambridge, Mass.) overnight at 4°C blocked by following with 1% gelatin (enzyme immunoassay grade; Bio-Rad) for 1 h at 37°C. After the washing of each well, urine specimens diluted with the sample dilution buffer were allowed to react for 1 h at 37°C. After the washing of the antibody-gold conjugate was stopped by the addition of 4 M NaOH solution, the absorbance of each well at 405 nm was measured with a microplate reader (EL 312e; Bio-Tek Instruments, Winooski, Vt.).

RESULTS

Polyclonal and monoclonal antibodies to LF. The polyclonal antibody and 14 monoclonal antibodies to LF did not cross-react with transferrin or bovine LF when examined by an ELISA method with these antigens immobilized in the wells of microtiter plates. Moreover, the two monoclonal antibodies, 17B04-08 and 32D01-10, used for the IC test strip were shown to have specificities for different epitopes on the LF molecule when examined by a sandwich ELISA.

Sandwich ELISA of LF. To quantitatively assess the detectable range of LF, standard solutions of LF at concentrations from 411.5 pg/ml to 300 ng/ml were examined. As shown in Fig. 1, a well-fitted standard curve was obtained (r = 0.998; P < 0.001). The quantitative detection range of LF was from 4.8 to 117 ng/ml (n = 6; P < 0.001).

Normal urine specimens fortified with various amounts of LF (0, 25, 50, 100, 200, and 300 ng of LF/ml of urine) were diluted 1:5 and examined to evaluate the LF recovery rates throughout the procedure. As summarized in Table 1, the mean recovery rates obtained with each level of added LF ranged from 88.4 to 124.8%, with standard deviations (SD) of from 5.0 to 26.4%.

We examined 121 urine specimens from apparently healthy
persons, 28 from patients without UTI, and 60 from UTI patients (33 with uncomplicated UTI and 27 with complicated UTI). The results are summarized in Table 2 as the mean (± SEM) urinary LF concentration. No significant difference in LF concentration was observed between the uncomplicated and complicated UTI populations. Therefore, the populations were combined as the UTI-positive population for the purpose of this study. We compared the LF concentrations in specimens from the healthy population with those from UTI-negative and UTI-positive populations. There was no significant difference between the healthy and UTI-negative populations, whereas the difference between the UTI-negative and UTI-positive populations was significant (P < 0.001). The urinary LF concentration of the UTI-positive population was more than 50-fold higher than that of the UTI-negative population. These results suggest that an excessive amount of LF was released from PMNs into the urine by inflammatory processes.

To determine the minimum detectable concentration of LF, various amounts of standard LF (0, 20, 50, 100, and 200 ng and 100 µg of LF/ml) were examined. The line produced by capturing the antibody-gold conjugate was observed at the control zone of all test strips, suggesting that the antibody-gold conjugate migrated by capillary action through the membrane beyond the detection zone without aggregation. Another line was revealed on the detection zone by the production of the sandwich immunocomplex of LF and two monoclonal antibodies when the concentration of LF was ≥50 ng/ml. In addition, the detectable concentration range extended to >100 µg/ml without false-negative results due to the prozone phenomenon. These results indicated that this IC system for the detection of LF has a minimum detectable concentration of 50 ng of LF/ml and a wide dynamic range.

The PMN lysate was examined with the IC test strip to detect LF released from PMNs. The results showed that LF in the lysate obtained from ≥10^7 PMNs/ml could be detected.

Each normal urine specimen containing <20 ng of LF/ml of urine fortified with various amounts of LF (0, 100, 200, 400, and 800 ng of LF/ml of urine) was diluted 1:4 and examined. The results showed that the detectable concentration of LF in the urine was 200 ng of LF/ml of urine.

The LF of each urine specimen quantified by ELISA was reexamined with the IC test strip. The results are summarized in Table 3 as a comparison of the presence of urinary LF with the results of microscopic examination of urinary PMNs and with the UTI diagnosis status. All 121 normal specimens were negative for LF, and 25 of the 28 PMN-negative specimens and 26 of the 28 UTI-negative specimens were assessed as negative based on the IC test strip results. Fifty-six of the 60 PMN-positive specimens and 57 of the 60 UTI-positive specimens were assessed as positive. The sensitivity, specificity, and predictive values of negative and positive findings of urinary LF in comparison with the results of urinary PMNs were 93.3, 89.3, 86.2, and 94.9%, and the values for predicting UTI were 95.0, 92.9, 89.7, and 96.6%, respectively. Thus, all indices of efficacy were very high, and the urinary LF examination results reflect the presence of urinary PMNs and predict UTI.

### DISCUSSION

In this study, we determined the feasibility of using urinary LF as a marker for UTI diagnosis and we evaluated the utility of a simple and rapid test for LF by IC. Because urine specimens contain many substances which interfere with the determination of LF, the analysis of LF in urine specimens has to be carried out in a detergent-rich sample dilution buffer, even though 0.1% Triton X-100 detergent solution alone can release LF from PMNs within 1 min (7).

In the preliminary study, the LF concentration in urine spec-
imens was quantified. For this purpose, we developed a sandwich ELISA with a rabbit polyclonal antibody to LF. The quantitative detection range of LF was from 4.8 to 117 ng/ml. Hetherington et al. reported that 10⁵ PMNs contain about 4.9 μg of LF (8). Therefore, this range requires about 5 × 10³ to 1.2 × 10⁵ PMNs/ml of urine and is sufficient to quantify LF in normal urine specimens. In UTI conditions, in which an excessive amount of LF is excreted from PMNs, the volume of the urine specimen to be analyzed can be reduced. The results of the recovery test for LF in normal urine specimens (recovery rates, 88.4 to 124.8%) indicate that the sandwich ELISA method is precise and accurate.

Furthermore, LF in the PMN lysate obtained from ≥10⁵ PMNs/ml could be detected by the IC test strip. This PMN density corresponds to about 50 ng of LF/ml, and the results correlated well with the minimum detectable concentration of LF evaluated with the LF standard solutions. These findings indicate that the IC test strip can detect LF released from PMNs precisely.

For the diagnosis of UTI with LF as a marker, it is important to define the cutoff value of the urinary LF concentration for the separation of negative and positive results. To define the cutoff value, we calculated the mean ± 3 SD (SEM × √n) value using the results obtained from the healthy population measured by the sandwich ELISA. Although this value was calculated as 180.4 ng of LF/ml of urine, it is difficult to detect a difference of 20 ng of LF/ml of urine with the IC test strip after urine specimens are diluted before examination. Therefore, the cutoff value was defined as 200 ng of LF/ml of urine instead of 180 ng of LF/ml to minimize false-positive results. Based on this cutoff value and the minimum detectable LF concentration of the IC test strip, urine specimens should be diluted 1:4 with the sample dilution buffer before analysis. The LF concentration in the urine detectable by the IC test strip is well correlated with the minimum detectable concentration determined with standard LF and the cutoff value determined according to the results of the ELISA. This indicates that urinary LF can be detected precisely by the IC test strip without serious interference by other substances present in urine.

The LF concentrations in urine specimens from UTI patients were much higher than those in specimens from both patients without UTI and healthy subjects. When the cutoff value for detecting the presence of urinary LF was set at 200 ng of LF/ml of urine, the LF positivity and negativity correlated well not only with the microscopic examination results of PMNs but also with UTI diagnosis. Furthermore, LF is stable in urine specimens even after the structures of PMNs are destroyed. For example, our preliminary experiments showed that the residual rates of LF from a normal urine specimen which was fortified with 200 ng of LF/ml of urine were 113.8% after storage at 45°C for 3 days and 94.4% after three cycles of freezing and thawing. Therefore, it is not always necessary to specify the time of specimen collection and the storage temperature. In addition, interfering reactions caused by reducing agents or antibiotics, as observed in the leukocyte esterase activity test (19), are not a problem, because this detection system for LF is based on a specific antibody-antigen reaction. Indeed, all nine of the urine specimens which were collected after antibiotic medication and which were positive in the detection of LF, several methods, such as an ELISA (8) and a latex agglutination assay (7), have already been developed. Even though the ELISA can detect 3 ng of LF/ml quantitatively, this method is not recommended for the rapid diagnosis of UTI due to its complicated and time-consuming procedure. In the latex agglutination assay, the minimum detectable concentration of LF is 310 ng/ml. Therefore, it is impossible to detect the cutoff line of 200 ng of LF/ml of urine even if the urine specimen is examined directly without further dilution. The IC test strip has a minimum detectable concentration of 50 ng of LF/ml and is able to detect the cutoff line even after fourfold dilution of a urine specimen. In addition, all examination processes can be completed within 10 min after specimen collection.

In conclusion, urinary LF is a sensitive marker for the diagnosis of UTI caused by inflammatory pathogens. The IC test strip provides a useful tool for the simple and rapid diagnosis of UTI. Large numbers of urine specimens are processed daily for the overnight bacterial culture test to determine the inflammatory pathogens, even though the majority of test results are negative. And the microscopic examination of PMNs requires skill and experience on the part of the microscopist. Therefore, the use of the IC test strip to detect LF could greatly reduce the time, cost, and effort of routine urinalysis and could avoid unnecessary antibiotic medication of UTI-negative patients.

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