Localizing the Site of Hematuria by Immunocytochemical Staining of Erythrocytes in Urine

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We have further explored the immunocytochemical staining method to discriminate renal and nonrenal hematuria, reported by Abram and Laird (Am J Kidney Dis 1987;3:44-50). After fixation on slides with acetone, erythrocytes in urine were stained with antiserum against human Tamm–Horsfall protein. Reactions were made visible by using either a fluorescent second antibody or a biotinylated second antibody, avidin, and biotinylated horse radish peroxidase, producing an insoluble reaction product. The staining methods were validated with material from clinically diagnosed cases of hematuria of renal or nonrenal origin. In material from kidney transplantation patients, in samples from the catheter that were presumed to contain renal erythrocytes, 84.7% and 80.1% of the erythrocytes stained by the immunofluorescence and immunoperoxidase methods, respectively, whereas in samples from the catheter that were supposed to contain nonrenal erythrocytes, 9.3% and 13.1% of the cells stained. In a group of nontransplantation patients with various causes of renal hematuria, 87.3% and 89.8% of the erythrocytes in urine stained with the immunofluorescence and immunoperoxidase methods, respectively, whereas in samples from patients with hematuria of known nonrenal origin, 12.9% and 12.4% of the cells stained. Staining of erythrocytes in renal and nonrenal hematuria was significantly different (P <0.001) and better discriminated between renal and nonrenal hematuria than did inspection of the morphology of erythrocytes in urine.

Additional Keyphrases: biotin–avidin interaction · Tamm–Horsfall protein · uromodulin · peroxidase · urinalysis

One of the first things to be determined when a patient presents with hematuria is the site of origin of the condition: the kidney or the urogenital tract. This distinction has important implications for diagnosis and treatment. However, the choice of noninvasive methods, as are obviously preferred for investigative purposes, is limited; methods generally involve inspection of urine sediment, tests of kidney function, and investigations for the presence of proteinuria.

Urine sediment can be inspected for the presence of casts or for dysmorphic erythrocytes (1, 2). Casts, however, are not always present, even in renal hematuria (3, 4). Reliable investigation for the presence of casts, as well as dysmorphic erythrocytes, requires direct inspection of fresh specimens, which may be inconvenient. Although proteinuria and abnormal kidney function suggest hematuria of renal origin, these conditions are only indirect evidence of such. Obviously, convenient tests that can give direct evidence about the origin of hematuria are desirable.

Evidence has been presented that erythrocytes in hematuria of renal origin are coated with so-called Tamm–Horsfall protein (THP), in contrast to the erythrocytes in hematuria of nonrenal origin (5). THP, or uromodulin, lines the luminal surface of the ascending limb of Henle’s loop. An important protein constituent in urine, THP is the major protein constituent of casts because of its strong propensity to polymerize (6–8). THP has been demonstrated on the surface of erythrocytes in urine in various renal disorders by direct immunocytofluorescence staining, but no evidence has supported post-renal coating of erythrocytes by THP (5).

Thus, immunocytochemical staining of erythrocytes in urine may be an excellent tool for characterizing hematuria. Intending to apply the previous studies on THP-coating of erythrocytes to diagnoses, we have been looking for possibilities to adapt the direct immunocytofluorescence method to a more convenient method that could be useful in routine clinical chemistry laboratories. Here, we report the feasibility of indirect immunocytofluorescence and immunoperoxidase staining methods to distinguish renal from nonrenal hematuria and compare the results with those obtained by inspection of the erythrocyte morphology.

Materials and Methods

Patients and Sample Processing

Urine specimens were collected from patients seen between May 1990 and May 1991 at the Academic Hospital Nijmegen–St. Radboud and the Canisius-Wilhelmina Hospital, both in Nijmegen, The Netherlands. The patients were clinically diagnosed as having renal or urogenital diseases. With a few exceptions, as indicated, renal diseases were diagnosed by renal biopsy. Except for the kidney transplant patients, patients with renal diseases (n = 40) were ages two to 77 years (mean 21, SD 20); 32 of these were male. The kidney transplant patients (n = 25) were ages two to 60 years (mean 40, SD 19); 14 were male. The patients with urogenital diseases (n = 25) were ages seven to 84 years (mean 60, SD 18); 20 were male.

Hematuria was assessed by a dipstick test (Combur-9 RL; Boehringer Mannheim, Mannheim, F.R.G.). Hema-
turia was either the result of disease or was caused by medical intervention. We obtained from the kidney transplant patients two urine samples simultaneously, one via a catheter in the pelvis of the transplanted kidney and one via a catheter in the bladder. Samples with moderate hematuria (60–500 cells/µL) were processed as received. Samples without visible macroscopic hematuria (<50 cells/µL) and samples with massive macroscopic hematuria (>500 cells/µL) were centrifuged for 10 min at 2000×g to concentrate or dilute the cell suspension, respectively. Cells were resuspended at a concentration of about 100 cells/µL in a small volume of supernate, to avoid osmotic strain to the cells. Urine samples were inspected by microscopy and processed for immunocytochemical staining within 1 h.

To evaluate the staining methods, we stained erythrocytes from urine specimens from each patient by the immunofluorescence and the immunoperoxidase methods and also inspected their morphology. Only some erythrocyte samples that were too old for morphology inspection, that had too few cells remaining on the slides after staining, or that had been taken in the beginning of our studies when the immunoperoxidase staining method was not yet operational were not evaluated. Such specimens were thus investigated by only one or two of the methods.

**Inspection of Erythrocytes Morphology**

Erythrocyte morphology was evaluated according to the method of Fassett et al. (4) by trained observers who were uninformed of the patients' diagnoses. Essentially, they distinguished between (normal) isomorphic erythrocytes—which are biconcave discoid with a regular surface—and dysmorphic erythrocytes—which are oval, small, and have a surface with a disrupted appearance. Between 100 and 200 cells per urine specimen were counted in a Bürker counting chamber (Fortuna Graf, Wertheim, F.R.G.).

**Immunocytochemical Staining Methods**

For staining, a drop of cell suspension was applied onto glass microscope slides and allowed to evaporate at ambient conditions. Subsequently, the sample was fixed in acetone for 10 min and stored at -20°C until immunocytochemical staining.

Staining was performed at room temperature. The immunofluorescence staining method was as follows. Slides with fixed preparations were first rinsed with buffer A (20 mmol of citric acid and 40 mmol of Na2HPO4 per liter, pH 5.2), then incubated with 30 µL of anti-human THP antiserum, diluted 120-fold in buffer B (50 mmol/L Tris·HCl, pH 7.6) for 90 min. Controls, run simultaneously, were mixed with normal rabbit serum, diluted 120-fold in buffer B. All preparations were rinsed with buffer A and incubated for 60 min with normal human serum, 100 µL/L, diluted in buffer B. After rinsing the slides with buffer A, we added 30 µL of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum, diluted 180-fold in buffer B, and incubated these for 60 min, rinsed them with buffer A, and let them air-dry. To imbed the samples, we used a 1 g/L solution of p-phenylenediamine plus glycerol, 900 mL/L, in phosphate-buffered saline (pH 7.8). Microscopic inspection was done under ultraviolet illumination with a fluorescence microscope at a magnification of 100 × 10.

The immunoperoxidase staining method differed from the immunofluorescence staining method as follows. Before staining, we treated the preparations on the slides with 15 mL/L H2O2 reagent for 60 min to quench the endogenous peroxidase (EC 1.11.1.7) activity of the erythrocytes. From that point until the addition of FITC-conjugated antibodies, the immunoperoxidase method was the same as the immunofluorescence method. Then, instead of FITC-conjugated antibodies, we incubated the preparations for 30 min with 30 µL of biotinylated goat anti-rabbit antiserum, diluted 400-fold in buffer B. After rinsing the slides, we incubated them for 30 min in buffer B containing 200-fold-diluted avidin and 200-fold-diluted biotinylated horseradish peroxidase (diluted from the ABC kit from Vector Labs., Burlingame, CA; this mixture was prepared 30 min before use). Finally, the slides were rinsed again and incubated for 10 min with 30 µL of a solution of 0.5 g of diaminobenzidine and 0.3 mL of H2O2 per liter of buffer A. After rinsing the slides, we inspected them under a light microscope at a magnification of 1000.

**Reagents**

Rabbit anti-human THP antiserum was from Biomedical Technologies Inc., Stoughton, MA (obtained via Sanbio, Uden, The Netherlands); FITC-conjugated goat anti-rabbit IgG antiserum was from Kallestad, Austin, TX; biotinylated goat anti-rabbit antiserum was from the Vector Labs. ABC-kit; 3,3'-diaminobenzidine was from Sigma Chemical Co., St. Louis, MO; and normal rabbit serum and human serum were obtained in-house. All other chemicals were from Merck, Darmstadt, F.R.G.

**Results**

**Development of Immunocytochemical Staining Methods**

Our first option was to change the direct immunofluorescence staining method for THP (5) to an indirect immunocytochemical staining method. Furthermore, we wanted to use peroxidase instead of a fluorescence label to monitor the reaction, thus making the staining method more convenient for the routine clinical chemical laboratory.

We were unable to obtain reproducible staining by indirect immunocytochemical staining methods for erythrocytes in suspension, the type of sample used by Abrass and Laird (5), even though we followed the published method carefully. The reasons for this failure remain unknown. However, when we slightly adapted the method and fixed the cells on slides with acetone before staining, excellent results were obtained.

**Immunofluorescence**. Figure 1 shows the results of indirect immunofluorescence stains of erythrocytes in
urine of patients with renal hematuria, with anti-THP antibody as first antibody. Erythrocytes showed a bright green fluorescence along the circumference of the membrane surface and a less intense green interior, both against a dark-greenish background (Figure 1, a and b).

Sometimes the membrane fluorescence around the cells was regular, predominantly for erythrocytes in freshly voided urine. More often, however, the fluorescence on the cell surface contained local dots of more intense fluorescence. No staining of erythrocytes was observed when the anti-THP antibodies were omitted from the procedure and replaced by normal rabbit serum (not shown). Nor was staining observed for erythrocytes in urine when the hematuria was of nonrenal origin (Figure 1, c and d).

Occasionally, leukocytes and epithelial cells also showed membrane fluorescence, and bright fluorescent clumps of debris were regularly observed. The fluorescence of all these objects was less intense when the anti-THP antiserum was omitted or when the urine was from patients with nonrenal disease. This suggests some relationship of the staining with THP and renal disorder. However, we did not undertake a systematic study of this staining.

**Immunoperoxidase.** When acetone-fixed erythrocytes in a sample with hematuria of renal origin were stained with anti-THP antiserum plus a second antibody conjugated with horseradish peroxidase, only faint dark-brown rings were visible around the erythrocytes. This result was barely distinguishable from that for erythrocytes from nonrenal origin (data not shown). However, when the staining signal was amplified by use of a biotinylated second antibody, avidin, and biotinylated horseradish peroxidase, erythrocytes in samples from renal hematuria showed distinct dark-brown surfaces (Figure 2, a and b). These surfaces were stained rather uniformly. Compared with the fluorescence staining pattern, more details were visible; also, the cells were better differentiated from background, and intensely stained debris was rare. When the anti-THP antibodies were omitted from the procedure, the erythrocytes did not acquire brown surfaces (Figure 2c). In samples from patients with nonrenal disorders, only a few (if any) cells stained brown (Figure 2, d and e). Occasionally, leukocytes and epithelial cells in samples from renal

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Fig. 1. Micrographs of indirect immunofluorescence staining of erythrocytes in urine with rabbit anti-THP antiserum and FITC-conjugated goat anti-rabbit antiserum: (a) specimen from the catheter in the ureter draining the new kidney in a kidney transplant, (b) specimen from a patient after a renal biopsy, (c) specimen from the catheter in the bladder after a kidney transplant, and (d) specimen from a patient after transurethral resection of a bladder carcinoma. Pictures taken under ultraviolet illumination, at 600× magnification.

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Fig. 2. Micrographs of indirect avidin–biotin-amplified immunoperoxidase staining of erythrocytes in urine with rabbit anti-THP antiserum: (a) specimen from the catheter in the ureter draining the new kidney in a kidney transplant; (b) specimen from a patient with Alport syndrome; (c) specimen from a patient with Alport syndrome, when anti-THP antiserum was replaced by normal rabbit serum; (d) specimen from the catheter in the bladder after a kidney transplant; and (e) specimen from a patient after a transurethral resection of a bladder carcinoma. Pictures taken from a light microscope at 900× magnification.
patients showed dark-brown surfaces, whereas those from nonrenal patients did not. Again, no systematic study of the staining of these cells was made.

Validation of the Immunochimical Staining Methods

**Samples from kidney transplant patients.** The indirect immunofluorescence and the indirect biotin–avidin–amplified immunoperoxidase staining methods on acetone-fixed urinary erythrocytes were first validated with material from patients who had undergone a kidney transplantation. In such patients two catheters initially are present postoperatively, one in the pelvis of the transplanted kidney and one in the bladder. The former catheter provides urine with erythrocytes that originate from the kidney (renal erythrocytes), and the latter provides erythrocytes that originate predominantly from the urinary tract, notably, from the bladder incision and the lesions that resulted from connecting the ureter of the new kidney to the bladder (nonrenal erythrocytes). Although investigation of hematuria in transplant patients may not often be of clinical utility, in this way we easily acquired two types of hematuric urine samples simultaneously from one individual.

The results of the immunochimical staining of erythrocytes in urine from the two types of catheters in kidney transplant patients are summarized in Figure 3. The number of dysmorphic erythrocytes in the samples is also given. For various samples collected from the catheter in the pelvis of the transplant patients, the immunofluorescence method stained an average of 84.7% (SD 11.9%; n = 19) of the erythrocytes, whereas the immunoperoxidase method stained 80.1% (SD 6.9%; n = 12); 46.9% (SD 21.3%; n = 18) of the erythrocytes in these samples were dysmorphic. The average percentage of erythrocytes stained in samples from the catheter draining the bladder of the kidney transplants was 9.3% (SD 16.7%; n = 22) and 13.1% (SD 16.1%; n = 10) by the immunoperoxidase method; 27.4% (SD 14.2%; n = 18) of the erythrocytes in these samples were dysmorphic.

Clearly, the erythrocytes in urine of renal origin were well distinguished from the erythrocytes of nonrenal origin, both by immunofluorescence and immunoperoxidase staining for THP. For both methods the difference in numbers of stained cells for the two types of hematuria was significant (P < 0.001; one-sided Student's t-test). The difference becomes even more pronounced if the exceptional results for patient no. 2 (Figure 3) are omitted. The relatively high number of staining cells in this sample from the bladder drain might well have resulted from the passage of renal erythrocytes from the transplanted kidney along the outside of the catheter to the bladder. Identification of dysmorphic erythrocytes distinguished renal and nonrenal hematuria less well (P < 0.005) than did immunochimical staining.

**Samples from patients with renal and nonrenal disorders.** The immunochimical staining methods were also evaluated with specimens from patients previously diagnosed with renal or urogenital disorders, including several cases in which the bleeding resulted from medical interventions in the kidneys or the urogenital tract. The results of the immunochimical staining and morphological inspection of erythrocytes in the samples of the different clinical groups are compiled in Table 1. The findings were in line with the results obtained with the samples from the kidney transplant patients. Most erythrocytes in samples from patients with overt renal hematuria were immunochimically stained with the anti-THP antiserum, and many were found to be dysmorphic. Staining was absent when anti-THP antisera was replaced with normal rabbit serum.

Two cases of percutaneous nephrolithotripsy presented ambiguous results. The erythrocytes in urine samples from these patients stained faintly, but distinctly positive, by the peroxidase method, whereas the fluorescence method gave staining repeatedly judged as negative. Clinically, however, it is not clear whether erythrocytes appearing in the urine in these patients may be expected to be coated by THP, because the bleeding may originate from the renal parenchyma, damaged by the catheter insertion or from the renal.

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Fig. 3. Results of the immunocytochemical staining and of the inspection of the morphology of erythrocytes in urine of patients having undergone a kidney transplantation.

Urine specimens were obtained from a catheter in the ureter (R, renal), draining the transplanted kidney, or from a catheter in the bladder (NR, nonrenal). In most cases, the specimens were investigated by each of the following three methods: indirect immunofluorescence against THP (IF), indirect avidin–biotin–amplified immunoperoxidase staining against THP (IP), and relative number of dysmorphic erythrocytes (M). Some of the individual cases are identified by numbers for comparison of the results obtained with the different methods (if the number is not present in one of the columns, this determination was not performed on that sample). This illustrates that there is no straightforward relationship between the responses by each of the different methods.
pelvis, damaged by the kidney stones. In the latter case, erythrocytes do not pass through the tubules and would not be expected to be coated by THP; in the former case, however, they would. On the other hand, perhaps in this kind of renal bleeding, erythrocytes are coated with only a little THP. Because immunoperoxidase staining is more sensitive than is immunofluorescence staining, the two staining methods would produce different results, as was actually observed (Figure 4).

Various types of damage in the urogenital tract did not lead to positive immunochromical staining. Only in the beginning of our studies did we obtain a few outliers with the immunofluorescence method: significant numbers of erythrocytes were stained in two cases of transurethral resection of bladder carcinoma and in one case of transurethral resection of the prostate. These patients did not show signs of kidney disease at the time of the investigation and for at least half a year thereafter. Because in later studies we obtained no more exceptional results for samples from nonrenal disorders, we suppose the aforementioned observations to be related to our initial inexperience with the staining technique.

The pooled data for diverse renal and nonrenal disorders are represented in Figure 4. The immunofluorescence method stained 87.3% (SD 9.9%) of the erythrocytes in samples of renal hematuria (n = 35), and the immunoperoxidase method stained 89.8% (SD 7.6%; n = 16); 69.8% (SD 26.7%; n = 32) of the erythrocytes in these samples were dysmorphic. (The data for two cases of percutaneous nephrolithotripsy were excluded.) For samples of nonrenal hematuria, the immunofluorescence method stained 12.9% (SD 24.0%; n = 28) of the erythrocytes and the immunoperoxidase method stained 12.4% (SD 10.5%; n = 14). The proportion of dysmorphic erythrocytes in these samples was 15.8% (SD 12.9%; n = 20). Immunofluorescence and immunoperoxidase staining of erythrocytes, and also identification of dysmorphic erythrocytes, well discriminated renal from nonrenal hematuria in various disorders (P<.001; one-sided Student's t-test).

Discussion

Relatively few noninvasive tests are available for investigating the origin of hematuria. This inconvenient diagnostic situation would be aided significantly by new methods, such as might emanate from the findings of Abrass and Laird (5), who presented evidence that in renal hematuria the erythrocytes in urine were...
coated with THP, whereas in nonrenal hematuria they were not. Still, this study has not been followed by many others, perhaps because the technology described proved to be less convenient than expected. We have developed more amenable methodology, have demonstrated how this can distinguish renal and nonrenal hematuria, and have compared this method with another proposed to distinguish these types of hematuria.

The first technical modification to the previously published method (5) was to change from staining the cells in fluid phase to staining the cells dried and fixed on glass slides. Although of minor practical impact, this change proved very important because we were unable to obtain positive staining of cells in suspension. A possible consequence of this is that analysis of staining results by flow cytometry may not be feasible. One reason why we were unable to stain erythrocytes in suspension may be that, in the commercial antiserum used, the anti-THP antibodies were of lower titer than in the preparation used by Abrass and Laird, which was raised in-house and purified by affinity chromatography (5). Because reagents are diluted less for staining cells fixed on slides than for staining cells in suspension, a low titer would present less of a problem in the former situation. A second factor we suppose to have been important for the positive staining result of cells on slides may be the acetone fixation, which might preserve the antigenic determinants of THP on the cell surface. Presumably, THP will more easily be lost from unfixed cells in suspension.

A second modification was a change from direct to indirect immunocytochemical staining methods. This makes possible the use of unmodified first (anti-THP) antibody in combination with conjugated second antibody to monitor the reaction. Both kinds of antibodies are commercially available, in contrast to the conjugated anti-THP antibody used by Abrass and Laird (5). For the indirect immunoperoxidase method to be feasible, amplification was required; we used the avidin–biotin method for this. This modification made the result of immunoperoxidase staining superior to that of immunofluorescence staining. Immunoperoxidase staining produced more cell surface detail than did immunofluorescence, appeared to be somewhat more sensitive, and resulted in less staining of background and debris. Moreover, the results of immunoperoxidase stains can be viewed with a light microscope without diminution of the signal. Given the availability of light microscopes in clinical chemical laboratories, staining with immunoperoxidase thus would probably be preferred.

The clinical evaluation of the test for THP-coating of erythrocytes in urine shows that on the basis of the relative number of stained cells, good discrimination is possible between renal and nonrenal hematuria. Complete distinction between both groups was possible by immunoperoxidase staining of erythrocytes and apparently also by immunofluorescence staining. Staining cells in suspension, Abrass and Laird (5) found several cases of renal hematuria in which relatively few erythrocytes were stained with the anti-THP antiserum. As a result, the numbers of stained cells in renal and nonrenal hematuria showed some overlap. Except for the outliers in the beginning of our studies, as already mentioned, we did not encounter any overlap between the number of staining cells in renal and nonrenal hematuria. Thus, the discrimination between renal and nonrenal hematuria we observed was at least as good as that observed previously (5).

Immunocytochemical staining also gave sharper distinction between renal and nonrenal hematuria than did inspection of the morphology of the erythrocytes in urine. In both renal hematuria and nonrenal hematuria, significant numbers of dysmorphic and isomorphic erythrocytes can occur (4, 9, 10). Moreover, the erythrocyte morphology appears to be sensitive to the quality of the urine sample. Isomorphic erythrocytes were substantially distorted after incubation for 3 h in urine at room temperature (unpublished observations), whereas the immunocytochemical staining pattern was hardly affected. In addition, the distinction between dysmorphic or isomorphic erythrocytes appears to be somewhat arbitrary, although standardization within the laboratory is possible. A wide variation of the cutoff values used to discriminate renal from nonrenal hematuria has been reported, ranging from the extremes of about 20% to 80% dysmorphic erythrocytes (4, 9–11).
The main advantages of the morphological inspection of erythrocytes in urine are of course its relative ease and speed. Nevertheless, although immunochemical staining for THP is more laborious, its clinical performance is so much better that we believe it might be the test of choice.

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