Antigen and antibody detection assays for the diagnosis of tuberculosis

Pereira Arias, L.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 8

Detection of Immunoglobulin G Antibodies to Mycobacterium tuberculosis in Urine: Their likely Source and possible Role in Diagnosis of Tuberculosis

Lenka M. Pereira Arias – Bouda¹,²*, Sjoukje Kuijper¹, Lan N. Nguyen³, Henk M. Jansen³, and Arend H. J. Kolk ¹

KIT (Koninklijk Instituut voor de Tropen / Royal Tropical Institute) Biomedical Research, Amsterdam, the Netherlands,¹ Division of Pulmonary Diseases, Academic Medical Center, Amsterdam, the Netherlands,² The Pham Ngoc Thach Tuberculosis and Lung Diseases Center, Ho Chi Minh City, Vietnam³

Submitted for publication
Abstract

This study aimed to detect IgG antibodies against *Mycobacterium tuberculosis* in the urine of patients with tuberculosis, to investigate their possible role in the diagnosis of tuberculosis and their likely source (glomerular leakage or local production). Paired serum and urine samples were obtained from ten patients with pulmonary tuberculosis, nine patients with (pulmonary) diseases other than tuberculosis and ten healthy subjects. Two different sources of antigens were used, a Triton X-100 extract from *M. tuberculosis* (Ag360) and a short term culture filtrate from *M. tuberculosis* (Ag98). *Haemophilus influenzae* and *Streptococcus pneumoniae* bacteria were used as sources of control antigens. Serum and urine IgG antibodies were detected by ELISA against all four antigen preparations. The levels of IgG antibodies in serum against antigens in both *M. tuberculosis* preparations were significantly higher in the group of TB patients than in healthy subjects. However, it was not possible to discriminate between TB patients, patients with other diseases and healthy subjects using urinary IgG antibodies against either antigen source. The pattern of IgG antibody levels in serum against Ag360, Ag98, *H. influenzae* and *S. pneumoniae* was compared with the urine pattern. The urine patterns differed from the serum patterns in many cases, especially in those with TB. Urine/serum ratios of albumin, total IgG and anti-*M. tuberculosis* IgG were determined. Urinary IgG antibody levels to Ag360 and Ag98 were roughly 10-3 to 10-6 of serum levels. The relative coefficient of urinary excretion (RCE) of antibodies to Ag360 and Ag98 was defined as: [urine/serum ratio of IgG anti-Ag360 or Ag98] / [urine/serum ratio of total IgG]. For both antibodies (anti-Ag360 or Ag98) the mean RCE was ≤ 1 in all three groups, indicating that there was no local production of these anti-*M. tuberculosis* IgG antibodies in the urinary tract.

Introduction

Infections induce a humoral response against the infecting microorganism, and diagnosis can often be confirmed by the detection of specific antibodies in body fluids. Most assays are based on the detection of antibodies in the serum. In some situations however, a test using detection of urinary antibodies would be preferable. Urine samples can be easily collected, resulting in high patient acceptance, increased safety and reduced costs. In tuberculosis, it would be useful to use urine for diagnosis in those who produce sputum samples with difficulty, e.g. children.
Specific urinary antibodies have been found in several viral (12, 15, 25, 29, 31), parasitic (21), and bacterial (2, 8, 16, 33) infections, including leprosy (17, 18). Some of those urine tests have been shown to offer a useful alternative to diagnostic blood tests, for instance in *Helicobacter pylori* infection (16).

Antibodies against *M. tuberculosis* have been found in the serum of TB patients (reviewed in reference 5). Commercial assays are available, but are rarely used as the sole means of diagnosis because of unsatisfactory specificity and sensitivity (7, 19). Little is known about the presence of specific anti-*M. tuberculosis* antibodies in the urine from patients with pulmonary TB. Observations by our group indicated that specific IgG antibodies can be found in the urine of these patients (unpublished data). We were somewhat surprised to find that the urinary pattern of antibody levels in some patients differed substantially from the serum antibody pattern (e.g. patients TB1, TB2 and TB3 in Fig. 1). The question was whether the presence of these antibodies in the urine was due solely to glomerular leakage. If local antibody production contributed, then it would favourably influence the sensitivity and specificity of the urinary antibody test.

**FIG. 1.** Urine and serum antibody patterns in TB patients and non TB patients. ■, antibodies to 2 different *M. tuberculosis* antigens: a, Ag98 and b, Ag360. □, antibodies to other pathogens: c, *H. influenzae* and d, *S. pneumoniae*. The antibody levels were determined by ELISA as described in Materials and Methods. TB 1, Dutch adult: urinary antibody pattern differs from the serum pattern. TB 2, Dutch adult: although urinary and serum antibody patterns are comparable, in urine a shift is present towards antibodies against *M. tuberculosis*. TB 3, child from Zambia with primary TB: urinary antibody pattern differs from the serum pattern. TB 4, Dutch adult: urinary pattern is a reflection of serum pattern. Non TB 1-4: 2 Dutch adults (1 and 2) and 2 children from Zambia (3 and 4) with diseases other than TB: anti-*M. tuberculosis* antibody levels in serum and urine are clearly lower than in TB patients.
Immunoglobulins, mainly class G and to a lesser extent class A, are found in the urine from healthy subjects (26, 30) and patients with (non-renal) infectious diseases (6, 8, 13, 17). IgM is absent from normal human urine; its size prevents it from passing through the glomerular filter (27). IgA in normal urine is mainly of the secretory type (4). Some have thought that the urinary IgG is mainly produced within the renal tract (6, 24, 30). Others have speculated that the urinary IgG antibodies are mainly plasma-derived and excreted in the urine by glomerular leakage and transudation from blood capillaries along the mucosal epithelium of the urinary tract (8, 18). Recently, Tencer and co-workers (27, 28) showed that the IgG is filtered through size- and charge-selective large pores of the glomerular membrane.

The present study was done to determine whether specific anti-\textit{M. tuberculosis} IgG antibodies are detectable in the urine from patients with pulmonary tuberculosis, and, if so, whether their detection could be of value for diagnostic purposes. In addition, we tried to determine the likely source of any such antibodies. We have included two sets of controls, those with pulmonary diseases but no tuberculosis, and a group of healthy subjects with no respiratory illness and no history of tuberculosis.

**Materials and methods**

**Patients and controls.** The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center (protocol MEC98/034), and the Ethics Committee of the TB Center in Ho Chi Minh City. All subjects gave permission for blood and urine sampling after written information was provided.

Paired serum and urine samples were obtained from ten patients with pulmonary tuberculosis (TB) from the Pham Ngoc Thach TB and Lung Disease Center, Ho Chi Minh City, Vietnam. The urine samples were collected randomly during the day. The diagnosis in these patients was based on detection of \textit{M. tuberculosis} in cultured sputum. None of the patients was suspected of tubercular renal involvement. All patients were HIV-seronegative and had no previous history of TB.

Paired serum and urine samples from eight patients with pulmonary diseases other than TB were collected at the same Center. The urine samples were collected randomly during the day. Three patients suffered from pneumonia, three from chronic obstructive pulmonary disease and two had pulmonary malignancy. In addition, paired serum and urine samples were obtained from a patient with bacterial meningitis, from the same hospital in Vietnam. All patients were HIV-seronegative and only one patient had a history of TB, 20 years previously.
Detection of anti-M. tuberculosis IgG in urine

**TABLE 1. Albumin and total IgG concentrations in urine and serum**

<table>
<thead>
<tr>
<th>patient code</th>
<th>age (yr)</th>
<th>m/f</th>
<th>diagnosis</th>
<th>albumin</th>
<th>IgG</th>
<th>estimated IgG/albumin index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>urine (mg/l)</td>
<td>serum (g/l)</td>
<td>urine (mg/l)</td>
</tr>
<tr>
<td><strong>TB patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>42</td>
<td>f</td>
<td>pulmonary TB</td>
<td>1.6</td>
<td>43</td>
<td>1.2</td>
</tr>
<tr>
<td>T2</td>
<td>33</td>
<td>m</td>
<td>pulmonary TB</td>
<td>7.2</td>
<td>47</td>
<td>2.6</td>
</tr>
<tr>
<td>T3</td>
<td>41</td>
<td>m</td>
<td>pulmonary TB</td>
<td>54.7</td>
<td>39</td>
<td>10.1</td>
</tr>
<tr>
<td>T4</td>
<td>46</td>
<td>m</td>
<td>pulmonary TB</td>
<td>4.4</td>
<td>45</td>
<td>1.5</td>
</tr>
<tr>
<td>T5</td>
<td>26</td>
<td>m</td>
<td>pulmonary TB</td>
<td>4.0</td>
<td>43</td>
<td>2.3</td>
</tr>
<tr>
<td>T6</td>
<td>35</td>
<td>m</td>
<td>pulmonary TB</td>
<td>3.3</td>
<td>44</td>
<td>1.6</td>
</tr>
<tr>
<td>T7</td>
<td>28</td>
<td>f</td>
<td>pulmonary TB</td>
<td>10.6</td>
<td>44</td>
<td>3.9</td>
</tr>
<tr>
<td>T8</td>
<td>33</td>
<td>f</td>
<td>pulmonary TB</td>
<td>13.8</td>
<td>43</td>
<td>3.5</td>
</tr>
<tr>
<td>T9</td>
<td>36</td>
<td>f</td>
<td>pulmonary TB</td>
<td>14.3</td>
<td>43</td>
<td>7.4</td>
</tr>
<tr>
<td>T10</td>
<td>35</td>
<td>m</td>
<td>pulmonary TB</td>
<td>2.3</td>
<td>45</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>non TB patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>61</td>
<td>f</td>
<td>pulm. malignancy</td>
<td>12.0</td>
<td>36</td>
<td>6.6</td>
</tr>
<tr>
<td>N2</td>
<td>53</td>
<td>f</td>
<td>pulm. malignancy</td>
<td>6.6</td>
<td>40</td>
<td>2.9</td>
</tr>
<tr>
<td>N3</td>
<td>24</td>
<td>m</td>
<td>pneumonia</td>
<td>14.0</td>
<td>40</td>
<td>11.7</td>
</tr>
<tr>
<td>N4</td>
<td>69</td>
<td>f</td>
<td>COPD</td>
<td>5.1</td>
<td>32</td>
<td>2.5</td>
</tr>
<tr>
<td>N5</td>
<td>42</td>
<td>m</td>
<td>bronchitis</td>
<td>12.0</td>
<td>45</td>
<td>8.7</td>
</tr>
<tr>
<td>N6</td>
<td>80</td>
<td>f</td>
<td>pneumonia</td>
<td>4.6</td>
<td>26</td>
<td>2.8</td>
</tr>
<tr>
<td>N7</td>
<td>20</td>
<td>f</td>
<td>bact. meningitis</td>
<td>26.7</td>
<td>32</td>
<td>12.9</td>
</tr>
<tr>
<td>N8</td>
<td>72</td>
<td>f</td>
<td>COPD</td>
<td>10.9</td>
<td>35</td>
<td>7.1</td>
</tr>
<tr>
<td>N9</td>
<td>43</td>
<td>m</td>
<td>pneumonia</td>
<td>56.3</td>
<td>34</td>
<td>20.2</td>
</tr>
<tr>
<td><strong>healthy subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>42</td>
<td>m</td>
<td>-</td>
<td>10.9</td>
<td>42</td>
<td>2.7</td>
</tr>
<tr>
<td>H2</td>
<td>27</td>
<td>f</td>
<td>-</td>
<td>17.4</td>
<td>45</td>
<td>4.5</td>
</tr>
<tr>
<td>H3</td>
<td>47</td>
<td>m</td>
<td>-</td>
<td>8.0</td>
<td>43</td>
<td>2.5</td>
</tr>
<tr>
<td>H4</td>
<td>22</td>
<td>f</td>
<td>-</td>
<td>20.9</td>
<td>42</td>
<td>2.9</td>
</tr>
<tr>
<td>H5</td>
<td>30</td>
<td>f</td>
<td>-</td>
<td>371.0</td>
<td>39</td>
<td>13.5</td>
</tr>
<tr>
<td>H6</td>
<td>48</td>
<td>m</td>
<td>-</td>
<td>12.9</td>
<td>46</td>
<td>3.6</td>
</tr>
<tr>
<td>H7</td>
<td>34</td>
<td>m</td>
<td>-</td>
<td>18.4</td>
<td>48</td>
<td>2.6</td>
</tr>
<tr>
<td>H8</td>
<td>42</td>
<td>f</td>
<td>-</td>
<td>5.5</td>
<td>44</td>
<td>1.1</td>
</tr>
<tr>
<td>H9</td>
<td>36</td>
<td>f</td>
<td>-</td>
<td>5.3</td>
<td>47</td>
<td>1.2</td>
</tr>
<tr>
<td>H10</td>
<td>41</td>
<td>m</td>
<td>-</td>
<td>5.3</td>
<td>45</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Upper reference limit for the concentration of albumin in randomly collected urine in an adult healthy population is - 42 mg/l (Tencer et al., 1996). In this study micro-albuminuria was defined as 42-200 mg albumin/l and albuminuria as >200 mg albumin/l.

* Normal serum albumin levels: 35-50 g/l.

* IgG/albumin index: [urine/serum ratio of total IgG] / [urine/serum ratio of albumin]. Calculated values are an approximation of the real index, since concentrations of IgG and albumin were measured in randomly collected urine samples.

* pulm. malignancy: pulmonary malignancy. COPD: chronic obstructive pulmonary disease.
Paired serum and urine samples were collected from ten healthy volunteers from Amsterdam, The Netherlands. The urine samples were collected at random times during the day. None of these individuals had a past history of TB.

Patient characteristics are shown in Table 1. TB patients are numbered T1 to T10, patients with other diseases N1 to N9 and healthy subjects H1 to H10.

Storage of serum and urine samples. After collection, the serum samples were stored at -20°C in 2 ml-vials (Sarstedt, Nümbrecht, Germany). The urine samples were stored at 4°C in polystyrene 50 ml-vials (Greiner labortechnik, Nürtingen, Germany). Urine samples were not frozen, since we have evidence that freezing and thawing of urine causes false-positive reactions in antibody assays.

Preparation of Mycobacterium tuberculosis antigen mixtures Ag360 and Ag98. We used two sources of antigens: a Triton X-100 extract from a sonicate of *M. tuberculosis* cells (Ag360), and a culture filtrate from a short term culture of *M. tuberculosis* (Ag98). Ag360 and Ag98 were selected for this study based on the results with a serum panel of 40 Dutch TB patients and 40 Dutch healthy controls (for Ag360 specificity and sensitivity were 90% and 76%, respectively, and for Ag98 90% and 50%, respectively).

*Ag360. M. tuberculosis* (strain *M. tuberculosis* 1 (32)) was cultured in a protein-free medium (Sauton medium) for 3 weeks at 37°C. The suspension was centrifuged at 18,500 x g for 30 minutes at 4°C. The bacteria were washed twice with deionized water and stored at -70°C. Hundred ml of 0.5% Triton X-100 in 10mM Tris-HCl pH 8 was added to 75 g wet weight of *M. tuberculosis* bacteria. The bacteria were killed by heating the suspension at 56°C for 1 hour. Then, the extract was placed on ice and sonicated (Branson 250-Sonifier, Branson Ultrasonics Corporation, Danbury, Connecticut, U.S.A.) twice for 15 minutes at 4°C. After centrifugation of the sonicate for 30 minutes at 48,384 x g and 4°C, the supernatant was decanted and stored at 4°C. The pellet was resuspended in 100 ml extraction buffer and the extraction procedure was repeated twice as described above. The three supernatants were combined and centrifuged for 1 hour at 99,600 x g and 4°C. The supernatant was applied to a 1 ml-ExtractiGel D column as described by the manufacturer (Pierce, Rockford, U.S.A.) to remove the Triton X-100, and the protein concentration was measured using the BCA-Protein Assay Reagent (Pierce, Rockford, U.S.A.). The solution was stored in aliquots at -70°C.

*Ag98. M. tuberculosis* (strain *M. tuberculosis* 1) was cultured as described above. After centrifugation, the culture supernatant was filtered through a 0.2 μm filter and precipitated with 45% saturated ammonium sulfate at pH 4, and centrifuged again. The pellet was solubilized with 10 mM sodium phosphate and neutralized with 1M NaOH to pH 8, using phenolphthalein as pH indicator. Then, the solution was adjusted to 0.5 M ammonium sulfate and applied to a hydrophobic interaction column (Phenyl Sepharose High Performance
Detection of anti-\textit{M. tuberculosis} IgG in urine

column; Pharmacia), equilibrated with 0.5 M ammonium sulfate in 10 mM Na$_2$PO$_4$ pH 8.0. A stepwise elution was applied, the fraction eluted with 0.125 M ammonium sulfate in 10 mM Na$_2$PO$_4$ pH 8.0 was called Ag98.

**Sonicates of \textit{Haemophilus influenzae} and \textit{Streptococcus pneumoniae}**. \textit{Haemophilus influenzae} and \textit{Streptococcus pneumoniae} were cultured in brain heart infusion broth as previously described (22). The washed and killed bacteria were ultrasonicated for 5 minutes in a Branson 250-Sonifier.

**Detection of serum- and urinary IgG antibodies against \textit{M. tuberculosis} antigen preparations Ag360 and Ag98, and against other bacteria.** Serum- and urinary antibodies against Ag360, Ag98, \textit{S. pneumoniae} and \textit{H. influenzae} were detected by enzyme-linked immunosorbent assay (ELISA). Ag360, Ag98 and sonicates of \textit{S. pneumoniae} and \textit{H. influenzae} were coated onto polystyrene flat-bottom micro-titer plates (High Binding; Greiner labortechnik, Nürttningen, Germany) at a concentration of 5 µg of protein per ml in PBS pH 8.0. The plates were incubated overnight at 37°C in a water bath and washed 3 times with washing buffer containing 0.15 M NaCl, 1.2 mM KH$_2$PO$_4$, 4.8 mM Na$_2$HPO$_4$ and 0.05% (w/v) Tween 80, pH 7.3. After blocking the plates for 1 hour at room temperature (20°C), with 1% BSA in PBS pH 8.0, 150 µl/well, the plates were washed again 3 times and 100 µl of serum (dilution 1:500 in dilution buffer containing 0.1 M Tris, 0.15 M NaCl, 1% BSA and 0.05% Tween 80, pH 8.0.) or 100 µl of urine (pH adjusted by addition of 0.1 volume of 10 times concentrated dilution buffer, resulting in a dilution of the urine by a factor 1.1) was added to each well. All samples were tested in duplicate and paired serum and urine samples were tested on the same plate. The plates were incubated for 1 hour at 37°C in a water bath. After washing the plates 4 times, wells were filled with 100 µl of peroxidase-labeled goat anti-human IgG (Fcγ fragment specific) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) in a 1: 80,000 dilution in dilution buffer. The plates were incubated for 1 hour at 37°C in a water bath and washed again 4 times, followed by addition of 100 µl of tetra methyl benzidine (TMB) substrate solution (0.04% TMB, 0.04% urea-peroxide in 0.1 M sodium acetate citric acid buffer pH 4.0). After 30 minutes incubation in the dark at room temperature, the absorbance at 630 nm (A$_{630}$) was measured with a micro-titer plate reader (Bio-kinetics reader, Bio-tec Instruments, Winooski, USA). The reaction was then stopped by the addition of 100 µl of 0.5 M H$_2$SO$_4$ to each well, and the absorbance at 450 nm (A$_{450}$) was measured. By measuring at two wavelengths (630 nm and 450 nm), we were able to increase the detection range of the ELISA. High concentrations of specific IgG could be detected by measuring the A$_{630}$, low concentrations by measuring the A$_{450}$. The relation between the two is: $ \frac{A_{450}}{A_{630}} = 3 $. To control for the background reaction (conjugate control), for every coated antigen two wells were filled with sample dilution fluid instead of
patient serum or urine. The final results were expressed as the mean $A_{450}$ of the duplicates, after subtraction of the background-$A_{450}$. To correct for day-to-day and plate-to-plate variation, the same moderately positive serum sample was used as a positive control in every plate. All values were corrected by multiplying the $A_{450}$ of the unknown samples by the correction factor ($= A_{450}$ of the positive control serum at day 0/$A_{450}$ of the positive control serum at day of testing).

**Determination of the urine/serum ratio of specific IgG antibodies against Ag360 and Ag98.** The same ELISA method was used as described above for specific anti-bacterial IgG. Micro-titer plates were coated with Ag360 or Ag98. Serum samples were tested in a four-fold serial dilution from 1:500 to 1:2,048,000. Urine samples were pH adjusted by addition of 0.1 volume of ten times concentrated sample dilution buffer and tested on the same plate as the paired serum samples. The peroxidase-labeled goat anti-human IgG antibodies were used in a 1:8,000 dilution. The serum dilution which resulted in the same ELISA signal as was obtained using the (pH-adjusted) urine was determined for all samples (=calculated serum dilution factor, cSDF; see Fig. 2). The urine/serum ratio of IgG against Ag360 or Ag98 was defined as: $1/cSDF$.

**FIG. 2.** Determination of the urine/serum ratio of anti-*M. tuberculosis* IgG antibodies. A serial dilution of serum and the pH-adjusted urine (1.1× diluted urine, "undiluted urine") from the same patient were tested in ELISA for reactivity with *M. tuberculosis* antigens (Ag360 or Ag98). On the y-axis (log scale) the ELISA signal is shown, and on the x-axis (log scale) the dilution factor of the serum. At the right side the ELISA signal is shown of undiluted urine. From the serial dilution curve of the serum the dilution factor could be determined giving the same ELISA signal as undiluted urine (=cSDF, calculated serum dilution factor). The urine/serum ratio was defined as: $1/cSDF$.  

174
Quantitative measurement of total IgG in serum and urine and calculation of the urine/serum ratio. ELISA was used for measurement of total IgG in serum and urine. A polystyrene flat-bottom micro-titer plate (High Binding; Greiner labortechnik, Nürtingen, Germany) was coated with goat anti-human IgG (Fc\(_\gamma\) fragment specific) (Jackson ImmunoResearch Laboratories) at a concentration of 2 \(\mu\)g of protein per ml in PBS pH 7.2. The plates were incubated overnight at 37°C in a water bath and washed 3 times with washing buffer containing 0.05% Tween 80 in PBS pH 7.3. After blocking the plates for 1 hour at room temperature (20°C), with 1% bovine serum albumin (BSA) in PBS pH 8.0, 150 \(\mu\)l/well, the plates were washed again 3 times and 100 \(\mu\)l serum or urine (diluted in dilution buffer containing 0.1 M Tris, 0.15 M NaCl, 1% BSA and 0.005% Tween 20, pH 8.0) was added to each well. Serum was tested in a two-fold serial dilution starting from 1:200,000. Paired urine was tested on the same plate in a two-fold serial dilution starting from 1:200. Each dilution was tested in duplicate. The plates were incubated for 1 hour at 37°C in a waterbath. After washing the plates 4 times, wells were filled with 100 \(\mu\)l of peroxidase-labeled goat F(ab')\(_2\) fragments anti-human IgG (Fc\(_\gamma\) fragment specific) (Jackson ImmunoResearch Laboratories) in a 1:80,000 dilution in dilution buffer. The plates were incubated for 1 hour at 37°C in a water bath and washed again 5 times, followed by addition of 100 \(\mu\)l of TMB substrate solution (see above). After 30 minutes incubation in the dark at room temperature, the reaction was stopped by the addition of 100 \(\mu\)l of 0.5 M \(\text{H}_2\text{SO}_4\) to each well. The \(A_{450}\) was measured with a micro-titer plate reader. To control for the background reaction, one row was filled with sample dilution fluid. The final results were expressed as the mean \(A_{450}\) of the duplicates, after subtraction of the background-A\(_{450}\). In each plate a serial dilution of human IgG whole molecule (Jackson ImmunoResearch Laboratories) was tested ranging from 0.2 ng/ml to 200 ng/ml. To determine the concentration of total IgG, the ELISA values of the unknown samples were compared with those obtained using known amounts of IgG in a standard curve.

The urine/serum ratio of total IgG was defined as: [total IgG in urine (mg/l)] / [total IgG in serum (g/l)].

Calculation of the relative coefficient of excretion (RCE). Since urine-to-serum ratios may depend on the variable water content of urine, we used the relative coefficient of excretion of antibodies from serum to urine, as described by Schoonbrood and colleagues (23) for sputum proteins. The relative coefficient of urinary excretion (RCE) of IgG anti-Ag360 or IgG anti-Ag98 was defined as: [urine/serum ratio IgG anti-Ag360 or Ag98] \(\times\) 1,000 / [urine/serum ratio total IgG]. Multiplication by 1,000 was necessary, since total IgG in urine is given in mg/l and total IgG in serum in g/l.

Quantitative measurement of albumin in serum and urine and calculation of the urine/serum ratio. The concentration of albumin in serum was measured by
spectrophotometry with bromocresol green as reagent. The concentration of albumin in urine was measured by capture ELISA. A polystyrene flat-bottom micro-titer plate (High Binding; Greiner Labortecnik, Nütringen, Germany) was coated with rabbit anti-human serum albumin (anti-HSA) (DAKO A/S, Glostrup, Denmark) at a concentration of 2 μg of protein per ml in phosphate-buffered saline pH 7.2 (PBS). The plates were incubated overnight at room temperature (20°C) and washed 3 times with washing buffer containing 0.05% Tween 80 in Tris-buffered saline (TBS) pH 7.3. After blocking the plates for 1 hour at room temperature with 1% BSA in TBS pH 8.0, 150 μl/well, the plates were washed again 3 times and 100 μl of urine (diluted in 0.05% Tween 80 in TBS) was added to each well. Urine was tested in duplicate in a ten-fold serial dilution starting from 1:100. The plates were incubated for 1 hour at 37°C in a waterbath. After washing the plates 4 times, wells were filled with 100 μl of peroxidase-labeled rabbit anti-HSA (DAKO A/S, Glostrup, Denmark) in a 1:8,000 dilution in TBS containing 1% BSA and 0.05% Tween 80. The plates were incubated for 1 hour at 37°C in a waterbath and washed 5 times, followed by addition of 100 μl of TMB substrate solution (see above). The color reaction was measured as described above. To control for the background reaction, one row was filled with 0.05% Tween 80 in TBS. The final results were expressed as the mean A450 of the duplicates, after subtraction of the background-A450. In each plate a serial dilution of human serum albumin (HSA) (Sigma-Aldrich) was tested ranging from 5 ng/ml to 1000 ng/ml. To determine the concentration of HSA, the ELISA values of unknown urine samples were compared with those obtained using known amounts of HSA in a standard curve.

The urine/serum ratio of albumin was defined as: [albumin in urine (mg/l)] / [albumin in serum (g/l)].

Estimated IgG/albumin index. In this study, the concentrations of IgG and albumin were measured in urine samples which were collected randomly during the day. It is known that urinary excretion of proteins may fluctuate during the day. Because of this circadian rhythm of proteinuria the calculated IgG/albumin index in this study is an approximation of the index calculated from IgG and albumin concentrations in 24-hour urine. The estimated IgG/albumin index was defined as: [urine/serum ratio of total IgG] / [urine/serum ratio of albumin].

Statistical analysis. The Mann-Whitney U test was used to compare sets of analyses. \( P < 0.05 \) was considered significant. The relation between variables was analyzed by calculating the Pearson’s correlation coefficient (r).
Results

**Albumin and total IgG levels in serum and urine.** Table 1 shows the concentrations of albumin and total IgG in serum and urine from the study individuals. Albuminuria was found in one healthy control (subject H5), a remnant of pre-eclampsia from which the subject had suffered some months earlier. Micro-albuminuria was found in one TB patient (patient T3) and in one patient with pneumonia (patient N9); all others were normo-albuminuric according to the reference limits in randomly collected urine samples, as determined by Tencer and colleagues (1996). The urinary IgG level was significantly higher in patients with other diseases compared to TB patients or healthy controls \( (P < 0.05) \).

**Estimated IgG/albumin index.** In Table 1 the estimated IgG/albumin index is shown. The median values (and inter-quartile range) for TB patients, non TB patients and healthy subjects (with no signs of glomerular leakage) were 1.4 (1.3-2.2), 1.7 (1.3-1.9) and 1.1 (1.0-1.5), respectively. Although not statistically significant, the index in sick patients (with or without TB) was higher than in healthy subjects. A relatively high index was found in 3/10 TB patients (T1, T5 and T10: index 2.5, 2.7 and 2.2, respectively) and 3/9 patients with other diseases (N3, N4 and N8: index 2.1, 1.9 and 1.9, respectively). All healthy subjects had indices of 1.5 or lower. From the three individuals with (micro-)albuminuria, one had a selective proteinuria (H5: index 0.1). However, both albumin and immunoglobulin contributed to the proteinuria of patient T3 and N9.

**Levels of IgG antibodies to M. tuberculosis and other bacteria in serum and urine.** IgG antibodies against the *M. tuberculosis* antigen preparations Ag360 and Ag98 were detected in the sera from TB patients, non TB patients and healthy controls (Fig. 3a and b). The levels were significantly higher in the group of TB patients than in healthy subjects \( (P = 0.005 \text{ for IgG anti-Ag360 and } P = 0.03 \text{ for IgG anti-Ag98}) \). The differences between TB patients and patients with other illnesses were less evident. At an arbitrarily chosen cut-off point (mean antibody level + 1 SD of healthy subjects), 7/10 (70%) TB patients had IgG antibodies recognizing Ag360, compared to 4/9 (44%) patients with other diseases and 2/10 (20%) healthy subjects. Using the same definition for the cut-off point in the ELISA using Ag98, six of the patients with TB (60%) had IgG antibodies to Ag98 compared with 67% of patients with other illnesses and only 1 healthy subject. In contrast to anti-*M. tuberculosis* IgG serum levels, no significant differences in anti-*H. influenzae* IgG or anti-*S. pneumoniae* IgG serum levels were found between TB patients and healthy subjects (Figure 3c and d).

IgG antibodies to Ag360 and Ag98 were also detected in the urine from patients and controls (Fig. 3e and f). Urinary levels of IgG antibodies to Ag360 were high in two TB
FIG. 3. Levels of IgG antibodies to Ag360, Ag98, *H. influenzae* and *S. pneumoniae* in serum and urine from pulmonary TB patients, patients with diseases other than TB, and healthy subjects. The dots represent individual patients/subjects. The antibody level is expressed as the absorbance, measured at 450 nm (high values were converted from $A_{450}$ results, see Materials and Methods). Horizontal bars show an arbitrary chosen cut-off point at the mean antibody level +1 SD of healthy individuals. T2 and T7: TB patients; N8 and N9: patients with other diseases; H5 and H7: healthy subjects.
patients (T2 and T7); both patients also had high serum levels against these antigens. Interestingly, all TB patients had very low levels of IgG to Ag98 in urine. Of the individuals without TB, two subjects with increased glomerular leakage (N9 and H5) had the highest urinary IgG levels in their group against *M. tuberculosis* antigens, as well as against other bacteria, although their serum levels were only moderately high. Those with no signs of increased glomerular leakage, who had relatively high antibody levels in urine, also had high antibody levels in serum (e.g. patient T2, N8 and H7 in Fig. 3). It was not possible to discriminate between TB patients, patients with other diseases and healthy subjects using urinary antibodies against either Ag360 or Ag98.

FIG. 4. Examples of urine and serum antibody patterns from pulmonary TB patients, patients with other diseases and healthy subjects. IgG antibody level in urine (dilution factor urine: 1:1). IgG antibody level in serum (dilution factor serum: 1:500). (a)-(c), examples from patients where antibody patterns in urine differed from those in serum. (d)-(f), examples from individuals where antibody patterns in urine were a clear reflection of those in serum. Antibody levels are expressed as the absorbance, measured at 450 nm (high values were converted from *A*<sub>630</sub> results, see Materials and Methods). Ag98, Ag360, H. infl., and S. pneu., antibodies recognizing respectively Ag98, Ag269, *Haemophilus influenzae*, or *Streptococcus pneumoniae*. T2, T4, T8 and T10: TB patients; N3: patient with pneumonia; H8: healthy subject.
Comparison of IgG patterns in serum and urine. The pattern of IgG antibody levels against Ag360, Ag98, *H. influenzae* and *S. pneumoniae* in serum and urine were compared (Fig. 4). The urine patterns differed from the serum patterns in many cases especially those with TB (8/10), mostly due to a relatively low level of urinary antibodies to Ag360 and/or Ag98 (representative examples are given in Fig. 4a-c), but the differences in serum and urine patterns were less pronounced than observed in our earlier experiments (Fig. 1). However, in 5/9 non TB patients, 6/10 healthy subjects and in 2/10 TB patients (T3 and T4) the urine patterns were a clear reflection of the serum patterns (examples are shown in Fig. 4d-f).

Comparison of the excretion into urine of total IgG and IgG specific for the *M. tuberculosis* antigens Ag360 and Ag98. Urinary IgG antibody levels to Ag360 and Ag98 were roughly 10-3 to 10-6 of serum levels (data not shown). In all three groups a highly significant correlation was found between the urine/serum ratios of anti-Ag98 IgG and total IgG (Fig. 5). The same was found for anti-Ag360 IgG (data not shown). For both antibodies the median relative coefficient of urinary excretion (RCE) was < 1 in all three groups (Table 2), suggesting that there was no local production of these specific anti-*M. tuberculosis* antibodies in the urinary tract. Surprisingly, in TB patients, and to a lesser extent in patients with other diseases, the RCE of anti-Ag98 IgG was significantly lower than in healthy subjects (*P* < 0.001 and *P* = 0.01, respectively). The RCE of anti-Ag360 IgG was also lowest in TB patients, but only the difference between TB patients and healthy subjects was statistically significant (*P* < 0.05). Thus, the level of IgG antibodies to *M. tuberculosis* found in urine was lower than would be expected according to the urinary excretion of total IgG, especially in patients with active TB.

<table>
<thead>
<tr>
<th>group</th>
<th>n</th>
<th>RCE of IgG anti-Ag360</th>
<th>RCE of IgG anti-Ag98</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>10</td>
<td>0.15 (0.07-0.29)</td>
<td>0.23 (0.19-0.28)</td>
</tr>
<tr>
<td>non TB</td>
<td>9</td>
<td>0.23 (0.11-0.43)</td>
<td>0.37 (0.15-0.45)</td>
</tr>
<tr>
<td>healthy</td>
<td>10</td>
<td>0.31 (0.24-0.50)</td>
<td>0.74 (0.58-1.02)</td>
</tr>
</tbody>
</table>

*median values per group; ( ), inter-quartile range.*

180
Detection of anti-M. tuberculosis IgG in urine

Detection of anti-M. tuberculosis IgG in urine for TB patients, non TB patients, and healthy subjects. The correlation between the urine/serum ratio of total IgG and the urine/serum ratio of specific IgG antibodies to Ag98 is shown. On the x-axis (log scale) the urine/serum ratio of total IgG is shown: [urine (mg/l)] / [serum (g/l)]. On the y-axis (log scale) the urine/serum ratio of anti-Ag98 is shown (expressed as 1/cSDF), multiplied by 1000. Multiplication by a factor 1000 was necessary, since total IgG in urine is given in mg/l and total IgG in serum in g/l. The dots correspond to the individual patients/subjects. The slanted line represents an equal urine/serum ratio of specific anti-Ag98 IgG and urine/serum ratio of total IgG. Ur/se: urine/serum.

Discussion

Antigen 360 is a detergent extract of M. tuberculosis which contains a large variety of different antigens. Antigen 98 consists of a mixture of antigens secreted during the short term culture of M. tuberculosis. Thus both preparations contain a range of potential target antigens (both protein and lipopolysaccharide) against which antibodies produced in vivo during active tuberculosis could be directed. Whereas no differences in serum levels of antibodies against common bacteria such as H. influenzae or S. pneumoniae were found between Vietnamese patients with tuberculosis, Vietnamese patients suffering from other pulmonary diseases and Dutch healthy subjects, IgG antibodies recognizing elements in both M. tuberculosis mixtures (Ag360 and Ag98) were found more often in the serum of patients with pulmonary tuberculosis than in healthy Dutch people. However, similar antibodies were also found in other sick patients.

We were unable to discriminate between TB patients, patients with other diseases and healthy subjects in our tests with urine. IgG antibodies to secreted M. tuberculosis antigens (Ag98) were uniformly low in urine from patients with active tuberculosis. There was little correlation between the level of antibodies in the serum and their appearance in the urine. Individuals with increased glomerular leakage had relatively high urinary antibody levels against M. tuberculosis antigens as well as against other common bacteria, although their serum levels were only moderately high. However, all individuals who had high antibodies in
the urine but no signs of increased glomerular leakage, also had relatively high levels of antibodies against the same set of antigens in the serum. The antibodies to *M. tuberculosis* identified in healthy subjects and those without TB probably represent either cross-reacting antibodies, antibodies to antigens present in environmental mycobacteria or specific antibodies to *M. tuberculosis* in those who have been previously exposed to tuberculosis (as is likely in Vietnamese controls living in a TB endemic area).

In the urinary tract of healthy people, IgG antibodies enter the urine mainly by glomerular filtration (27). The importance of local production of urinary IgG in patients with infectious diseases is less clear. Published data on this subject are often contradictory (6, 8, 18, 24). It has been shown that the mucosal immune system of the urinary tract is capable of producing immunoglobulins during bladder infections, particularly IgG antibodies (10). Prentice and colleagues (20) have shown that the immunoglobulin production in the urinary tract can also be stimulated in response to an infection at another mucosal site, lending support to the concept of a common secretory immune system. This suggests that an infection in the respiratory tract could possibly stimulate local production of antibodies in the urinary tract of these patients. In the present study, the IgG/albumin index in patients with a respiratory infection was higher than the index in healthy subjects (Table I), although the differences were not statistically significant. Particularly in six of these patients a relatively high index was found (Table I: T1, T5, T10, N3, N4 and N8). Although no conclusions can be drawn from the absolute index-values, the indices in these six patients deviated strongly from those in healthy individuals and could indicate that a proportion of the urinary IgG in these patients is produced locally. However, in the majority of the patients no evidence was found for local production of IgG in the urinary tract.

During infections, bacterial antigens can circulate in the bloodstream and be excreted into urine (11, 14). Bentz and co-workers (3) found that 5% of patients with pulmonary TB had unanticipated positive urine cultures for *M. tuberculosis*. The presence of mycobacteria or mycobacterial antigens in urine could possibly evoke a specific mucosal immune response in the urinary tract. In support of this, we had found earlier that urinary antibody patterns in some TB patients did not reflect the serum antibody patterns (Fig. 1.: TB1, TB2 and TB3). However, in the present study, no such striking differences in urine and serum antibody patterns were found. The patients tested in our pilot (some shown in Fig.1.) were native-born Dutch adults in the Netherlands, or children in Zambia, whereas this study used samples from Vietnamese patients living in Vietnam. In this study, the relative urinary excretion of anti-*M. tuberculosis* specific IgG did not exceed the relative excretion of total IgG in any of the TB patients. Thus there was no evidence of local production of these specific antibodies.
The amount of anti-*M. tuberculosis* specific antibody appearing in the urine was lower than expected considering the total IgG excretion, especially in patients with active TB. A factor which could contribute to the low level of antibodies to *M. tuberculosis* in the urine is the IgG subclass distribution of the IgG antibodies to *M. tuberculosis* in serum. Although protein antigens mainly produce an IgG1 antibody response, repeated and long-term antigenic stimulation with T cell-dependent antigens (as in the patients from Vietnam) may lead to a marked IgG4 antibody response (1). Relatively fewer of these strongly anionic IgG4 antibodies than the neutral IgG1 antibodies will be transported across the negatively charged glomerular membrane (28). Also immune complex formation could contribute to the low level of urinary antibodies. In mice, monoclonal antibodies to the mycobacterium-specific lipopolysaccharide lipoarabinomannan form complexes which are cleared by the liver and which are not detectable in the urine (9).

Patients who suffer from (non-renal) infectious diseases often have a temporary increase in glomerular leakage (13). Any urinary antibody test using antigens which are not highly specific, will have problems because of the difficulty of determining the cut-off point. Negative control patients with increased glomerular leakage will also have increased amounts of (cross-reacting) antibodies in their urine, as was observed in some of the sick patients in the present study.

In summary, a test relying on the detection in urine of specific antibodies against *M. tuberculosis* to diagnose active pulmonary tuberculosis would theoretically be very helpful. We have used two different mixtures of *M. tuberculosis* antigens to provide a good chance of detecting such antibodies, but have been unable to develop a useful urinary test for tuberculosis. No evidence was found for local production of specific IgG antibodies against *M. tuberculosis* antigens in the urinary tract of Vietnamese adults with pulmonary tuberculosis.

**Acknowledgements**

L.M.P. was supported by a grant from the Amsterdam Society and Research Fund for Prevention and Cure of Tuberculosis.
References

Detection of anti-M. tuberculosis IgG in urine


