INHIBITORS IN URINE OF RADIOIMMUNOASSAY FOR
THE DETECTION OF GONOCCOCAL ANTIGENS

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THORNLEY, Wilson, Hormaeche, Oates and Coombs (1979) used a method of
solid-phase radioimmunoassay (RIA) devised for brucella antigens (Wilson,
Thornley and Coombs, 1977) to detect gonococcal antigens in a buffer system.
When this test was used to detect gonococcal antigens in urine samples, many
were found to be strongly inhibitory. This paper describes a study of some of
the properties of the inhibitors and ways of neutralising them.

MATERIALS AND METHODS

In general, materials and methods were as described by Thornley et al. (1979). Any
differences are set out below.

Urine samples from healthy men and men with non-specific urethritis or gonorrhoea were
as used previously (Thornley et al. 1979). Samples were also obtained from healthy women
members of the laboratory staff. Further samples from men and women were obtained by
courtesy of Dr J. G. Lines, Department of Clinical Biochemistry. These, referred to as
"biochemical-test urines" had usually been sent to the laboratory for assessment of the
subjects' renal function; the majority had normal renal function, and were free from genital-
tract infections. One urine sample (no. 5), obtained from the Public Health Laboratory,
Cambridge, was from a woman with cystitis. It was sterile when cultured but contained
about 75 000 pus cells/mm$^3$. The samples were stored at 5°C.

Preparation of urine supernatants. When stored at 5°C many of the samples formed
flocculent precipitates, which might have been expected to trap bacterial antigens. All were
kept at 37°C for 30 min. to allow as much precipitate as possible to dissolve and portions
were then centrifuged at 2000 g for 10 min. before the supernatants (SN) were separated.

Immunosorbent treatment of supernatants to remove immunoglobulins. To remove all
human immunoglobulins and especially potentially inhibitory antigonococcal IgG or IgA,
urine supernatants were treated with an adsorbent made by cyanogen bromide linkage
(Wide, 1969) of cellulose to rabbit anti-human myeloma IgG; this reacted also with IgA
through light-chain determinants.

The effectiveness of this reagent was tested by absorbing 10 ml of a 1 in 10 dilution of
normal human serum with 2 mg of the immunosorbent, with rotation of the mixture overnight
at room temperature. The absorbed serum was shown by haemagglutination inhibition to
contain less than 0.25% of the immunoglobulin originally present. The same method of
absorption was applied to 10-ml portions of urine supernatants, which were then separated
from the cellulose reagent before testing by RIA for inhibitory activity.

Fractionation of urine by dialysis and salt precipitation. Some urine samples were dialysed
for 2 days at 5°C against three changes of distilled water containing sodium azide 0.02% (w/v).
The inhibitory activity of dialysed urine was then either compared with that of an

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untreated sample of the same urine or the dialysed urine was further fractionated by precipitation with 0·58M sodium chloride overnight at 5°C, followed by centrifugation at 20 000 g for 15 min. The supernatant and the precipitate resuspended in water were dialysed against distilled water containing sodium azide 0·02% (w/v), and the material from the precipitate was centrifuged again to remove water-insoluble substances. Three fractions were obtained: one, soluble in 0·58M NaCl, a second, insoluble in 0·58M NaCl but soluble in water, and a third insoluble in 0·58M NaCl and in water. All were reconstituted to the same concentration as in the original urine before they were tested for inhibitory activity. This method of separation is based on the first step in the method of preparing Tamm-Horsfall glycoprotein (Maxfield, 1966), which should appear in the second fraction.

**RIA methods.** In principle the method consisted of incubating the sample containing antigen with an immunosorbent consisting of cellulose and rabbit anti-gonococcal IgG, to allow the capture of soluble antigen, followed by centrifugation and washing. The washed pellet was then incubated with radioactively labelled anti-gonococcal IgG, centrifuged, washed, and the bound radioactivity was counted. Details of the reagents and methods used to test sediments from 4-ml portions of urine are given by Thornley et al. (1979).

**RIA methods for testing urine supernatants.** These differed slightly in detail from the methods used for urine sediments, and are described below and in table 1. **Stage 1** was done

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Constituents of the mixtures in each stage of the radioimmunoassay for urine supernatants</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test</th>
<th>Contents of tubes during</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>pre-treatment</strong></td>
</tr>
<tr>
<td></td>
<td><strong>1 h incubation</strong></td>
</tr>
<tr>
<td>All</td>
<td>...</td>
</tr>
<tr>
<td>Specific tests</td>
<td></td>
</tr>
<tr>
<td>urine SN</td>
<td>...</td>
</tr>
<tr>
<td>urine SN and Gc standard</td>
<td>...</td>
</tr>
<tr>
<td>Gc standard</td>
<td>...</td>
</tr>
<tr>
<td>pre-treated urine SN</td>
<td>Urine SN (STI 1 ml); <em>either</em> (10 ml); <em>or</em> Pms-F in ethanol (0·5 ml)</td>
</tr>
<tr>
<td>pre-treated urine SN and Gc standard</td>
<td>Urine SN (10 ml); additions as above</td>
</tr>
<tr>
<td>Gc standard for comparison with above</td>
<td>PBS (10 ml); Gc standard (25 μl); <em>either</em> STI (1 ml) <em>or</em> Pms-F (0·5 ml) <em>or</em> ethanol (0·5 ml)</td>
</tr>
</tbody>
</table>

SN = Supernatant fluid; Gc = gonococcal; PBS = phosphate-buffered saline; STI = soybean trypsin inhibitor; Pms-F = phenyl-methylsulphonyl fluoride.
INHIBITORS OF RIA OF GONOCOCCAL ANTIGENS

in plastic screw-capped tubes (Twl 1 8 oz, Sterilin, Teddington). Portions of 10 ml of urine supernatant (SN) were mixed with 2.5 ml of assay buffer containing 0.125 mg/ml of the cellulose-antibody and, when required, 25 μl of a standard gonococcal antigen. Results were compared with those of standard gonococcal antigen in 10 ml of phosphate-buffered saline (PBS). All tubes were incubated with rotation for 17 h at room temperature. From each sample, duplicate LP3 tubes (Luckham Ltd, Burgess Hill) were prepared, each containing the pellet obtained by centrifuging 5 ml of the stage 1 mixture at 2000 g for 3.5 min. The supernatants were discarded and the pellets were washed three times with 1.5 ml of assay buffer. Stage 2: the washed pellet was resuspended in assay buffer and normal rabbit serum, to which 125I-labelled anti-gonococcal IgG was added. After rotation for 3 h, centrifugation and washing as in stage 1, the radioactivity bound to the pellet was counted.

In some experiments 10-ml portions of urine SN with various additives were rotated for 1 h at room temperature immediately before assay. The additives were 1 ml of a 10 mg/ml solution of soybean trypsin inhibitor (STI); or 0.5 ml of a 200mm solution of phenylmethylsulphonyl fluoride (Pms-F) in ethanol, or 0.5 ml of ethanol.

In some experiments, the results of the assay of gonococcal antigens were compared with the results of the assay of brucella antigens. The assays were done in the same way, except that cellulose coupled to anti-brucella antibody, a standard brucella antigen preparation, and labelled anti-brucella antibody were substituted for the corresponding gonococcal preparations. Details of the reagents for the brucella system are given in full by Wilson et al. (1977).

Calculation of results. Assay results for each sample were calculated as per cent. above background (Thornley et al., 1979). When the per cent. above background of the standard in the presence of urine (SU) was less than that of the standard in buffer (SA) the percentage inhibition was calculated from the expression:

\[
\frac{SA - SU}{SA} \times 100
\]

Urine samples were regarded as inhibitory when their percentage inhibition was greater than 15. The value for percentage inhibition was calculated only when the urine tested alone gave a very low result. When additives were used with the urine supernatant and standard gonococcal antigen, the corresponding value for the standard gonococcal antigen, assayed with the same additives in PBS, was used in the calculation.

Testing the influence of pus cells on the assay. To test the influence of pus cells on the assay when other urine constituents were highly diluted, a sterile urine sample (no. 5) containing 75 000 pus cells/mm³ was used. After storage at 5°C for 50 days the supernatant was prepared, and dilutions of 1 in 20, 1 in 200, and 1 in 2000 in distilled water were made from it. The original pus-cell count of the 1 in 20 dilution corresponded to that of a urine containing 3800 pus cells/mm³ and was close to the highest count observed in any of the Gc urines. At the same time, after a small portion of the original sample had been treated with ultrasonic vibration to break up all the remaining pus cells, the supernatant from this was prepared and diluted in the same way. Portions of 10 ml of each diluted supernatant were tested for inhibitory activity on the assay of standard gonococcal antigen.

RESULTS

When supernatants from NSU or Gc urines were tested in the assay without additives, the radioactivity bound was always less than 250% above background and usually less than 100% above background. The mean value for 19 supernatants from Gc urines was 59%, and 40% for 14 supernatants from NSU urines. It was assumed, therefore, that most of this binding was non-specific.

Urine supernatants and sediments inhibited the assay of added standard gonococcal antigen. Because measurement of the amount of inhibition depended
TABLE II

Inhibition caused by urine supernatants on the radioimmunoassay of added standard gonococcal antigen and the effects of adding soybean trypsin inhibitor

<table>
<thead>
<tr>
<th>Type of urine</th>
<th>Number of samples</th>
<th>Addition to test</th>
<th>Per cent inhibition Range</th>
<th>Mean</th>
<th>Number (%) of inhibitory samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSU</td>
<td>27</td>
<td>None</td>
<td>0-71</td>
<td>30</td>
<td>18(67)</td>
</tr>
<tr>
<td>Gc</td>
<td>33</td>
<td>None</td>
<td>0-79</td>
<td>28</td>
<td>22(67)</td>
</tr>
<tr>
<td>NSU</td>
<td>11</td>
<td>None, STI</td>
<td>0-46</td>
<td>19</td>
<td>6(55)</td>
</tr>
<tr>
<td>Gc</td>
<td>15</td>
<td>None, STI</td>
<td>0-75</td>
<td>30</td>
<td>12(80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-65</td>
<td>16</td>
<td>6(40)</td>
</tr>
</tbody>
</table>

* Inhibitory samples were those giving inhibition >15% in relation to the control.
NSU = non-specific urethritis; Gc = gonococcal; STI = soybean trypsin inhibitor.

on comparing the result of assaying the standard antigen in buffer with that of assaying it in urine, the inhibitory activity of many urine sediments could not be measured because the sample alone bound appreciable amounts of radioactivity. The inhibitory activity of urine supernatants was, therefore, studied in the first instance after the addition of standard gonococcal antigen which when assayed in buffer gave counts of about 1000% above background, i.e., very large counts compared with the values obtained with urine supernatants alone.

Table II lists the number of urine supernatants that inhibited the assay of standard gonococcal antigen by more than 15%. Of 27 supernatants from NSU urines, 18 (67%) were inhibitory at this level as were 22 (67%) of 33 supernatants from Gc urines. The range of inhibitory activity was also similar, ranging from 0 to 71% with a mean of 30% for samples from NSU urines, and from 0 to 79%, with a mean of 28%, for samples from Gc urines.

Inhibition due to proteolytic enzymes

It was suspected that inhibition might be due to proteolytic enzymes from pus cells. When the influence of pus cells on the assay was tested, with other urine constituents highly diluted, the 1 in 20 and 1 in 200 dilutions of sample no. 5 were inhibitory and the 1 in 2000 dilution was slightly inhibitory when the sample had been treated with ultrasonic vibration (fig. 1). Thus, some inhibitory activity remained in the pus cells in this specimen, even though it had been refrigerated at 5°C for 50 days.

When either soybean trypsin inhibitor (STI) or phenylmethylsulphonyl fluoride (Pms-F), both inhibitors of serine proteases, were added to the sample containing the 1 in 20 dilution of urine no. 5 supernatant, its inhibitory activity was completely abolished, suggesting that the inhibition caused by pus cells was due to proteases.

As a result of these findings, urine supernatants from NSU and from Gc samples were tested for inhibitory activity with and without added STI (table II).
INHIBITORS OF RIA OF GONOCOCCAL ANTIGENS

After treatment with STI those that were inhibitory when untreated either remained inhibitory, became less inhibitory, or lost all their inhibitory activity. Thus, in the presence of STI, only two of 11 NSU urines and six of 15 Gc urines remained inhibitory.

Supernatants from some non-infected urines also inhibited the assay of standard gonococcal antigen urine samples (C and 2Z, fig. 2). These two samples differed in their response to STI: adding it abolished the inhibitory activity of urine sample C but reduced only slightly that of sample 2Z.

Other inhibitory substances

It was clear that some urines contained other inhibitors not neutralised by STI. Further experiments showed that these were of at least two kinds. Thus, the inhibitory activity of some samples was reduced by dialysis. Heat-resistant inhibitors were found in some urines, and these may also have been dialysable; unfortunately, because of lack of material, the two tests were not done on the same urine samples. In samples that had been dialysed and precipitated with 0.58M sodium chloride, the STI-sensitive inhibitors remained in the salt-soluble fraction 1. Fraction 1 from two urines also contained inhibitors that were
STI-resistant, were not removed by treatment with the adsorbent designed to remove human immunoglobulins, and that, in the one sample so tested, were heat sensitive. It is possible that these inhibitors were proteases unaffected by STI. Fraction 2, containing Tamm-Horsfall glycoprotein, was never inhibitory. The inhibitory activity of several samples was reduced after treatment with adsorbent to remove human immunoglobulins, but the reduction was small. The largest change was from 55% inhibition to 35% inhibition, while several smaller reductions, such as that shown for urine 2Z in fig. 2, were found.

SitE of action of inhibitors

Several experiments indicated that inhibition took place mainly when the urine and standard antigen were incubated together. In one experiment, urine
samples were incubated with the cellulose-anti-gonococcal IgG adsorbent, which was then centrifuged and washed before the standard gonococcal antigen in buffer was added and assayed. The result of this test was compared with that obtained by the usual method when urine and standard gonococcal antigen were present together. Of eight samples that were inhibitory when assayed in the usual way only two showed any inhibition when first incubated with cellulose-antibody adsorbent, and it was less than the usual inhibition. This result implies that the inhibitors did not usually act by breaking down the antibody complexed to cellulose, nor did they block its site of attachment for antigen.

More conclusive results were obtained later when the inhibitory effect of the same urine samples on the assay of gonococcal antigen were compared with those obtained in assays of brucella antigen (fig. 2). Samples were chosen that, in the gonococcal assay: were not inhibitory, e.g., urine sample 10; or gave a slightly enhanced result, e.g., sample A; or had an inhibitory activity that was prevented by STI, e.g., sample C or whose inhibitory activity was slightly decreased, but not removed, by treatment with STI, immunosorbent or heat e.g., sample 2Z. None of these caused significant inhibition of the assay of standard brucella antigen (fig. 2). Thus, the two urines that inhibited assay of gonococcal antigen (samples C and 2Z) neither affected the cellulose-antibody adsorbent or the radioactive antibody, nor did they reduce the efficiency of the antigen-antibody reaction in the brucella system. It seems, therefore, that in the assay for gonococcal antigens, the proteolytic action of urines such as sample C must have been exerted against gonococcal antigens.

It is not clear how the other inhibitory urine, no. 22, inhibited the gonococcal-antigen assay system. Some inhibition could have been due to proteases and some to anti-gonococcal antibodies; some heat-resistant inhibition was present (38%). This unidentified substance also acts on the gonococcal antigen only, because the brucella assay was not inhibited.

Inhibitory activity of urine sediments

In parallel with the experiments described, the detection of gonococcal antigens in urine sediments was investigated (Thornley et al., 1979). The inhibitory activity of sediments was examined, as was that of supernatants, by comparison of results obtained when standard gonococcal antigen was added to the sediment with those obtained with standard antigen without urine but with all other additives. Urine samples from healthy men contained very few cells and 10 sediments from such samples bound very little radioactivity when tested alone. When standard gonococcal antigen and STI were added, inhibition was slight, the mean being 11% and the highest value 33%.

The sediments of three urine samples (including sample 2Z, fig. 2) the supernatants of which contained inhibitory substances resistant to STI and to heat, were strongly inhibitory, but this inhibition was completely neutralised by adding STI.
DISCUSSION

The inhibition by urine constituents of the radioimmunoassay of human growth hormone was found by Girard and Greenwood (1968) to be due to high concentrations of salts and urea, which could be removed by dialysis and which affected the efficiency of the antigen-antibody reaction. Some of the inhibitory activity found in the present study was also removed by dialysis, and may have been due to a similar cause.

However, in the present work other inhibitors, both STI-sensitive and STI-resistant, were found in dialysed urine supernatants. In experiments with the brucella antigen assay system, the efficiency of the antigen-antibody reaction was not affected by two urine supernatants whose inhibitory action on the gonococcal assay was due in one case to proteases and to unidentified heat-resistant substances in the other, suggesting that the inhibition produced by these samples on the gonococcal-assay system was due to their acting on gonococcal antigen. An additional implication of the results obtained with sample no. C (fig. 2) is that a large proportion of the gonococcal antigens detected by RIA are of protein nature.

Neutrophil polymorphonuclear leucocytes, which form the main part of the pus cells of urine samples, contain an elastase and a chymotrypsin-like enzyme (Dewald et al., 1975). The activity of similar, purified, enzymes from human spleen can be completely inhibited by STI and Pms-F (Starkey and Barrett, 1976a, b and c). The fact that the inhibition by many urine samples of the RIA of gonococcal antigens was prevented by STI indicates that it was probably due to the proteolytic action of either proteases from pus cells or to other proteases also sensitive to STI. Proteolytic action on the outer membrane proteins of the gonococcus could account for the decreased amount of antigen measured by the assay. It has been shown that some of these proteins are important antigenic determinants of the gonococcus (Johnston, Holmes and Gotschlich, 1976). In another gram-negative bacterium, Acinetobacter strain 199A, several major outer membrane proteins are hydrolysed by spleen elastase, while one is attacked by cathepsin G (Thorne, Oliver and Barrett, 1976).

The inhibitory activity of proteases was very marked in sediments from Gc or NSU urines, as could be expected from their content of pus cells, but was virtually eliminated by treatment with STI. The unidentified inhibitors found in the supernatants of several urine samples and resistant to STI and to heat, were not present in the sediments from the same samples. It was therefore considered satisfactory to use STI-treated urine sediments to detect gonococcal antigens by RIA, as described by Thornley et al. (1979), and trials of this method with standard gonococcal antigen added to each sample revealed only slight inhibitory action in 17% of the sediments.

SUMMARY

Several substances in urine were found to inhibit the radioimmunoassay of added gonococcal antigens. The supernatants of two-thirds of urine samples from male patients with either gonorrhoea or non-specific urethritis (NSU)
were inhibitory. The inhibition caused by many, but not all, samples was reduced or completely abolished by the addition of soybean trypsin inhibitor (STI); STI-sensitive inhibition is thought to be due to proteolytic enzymes, probably from pus cells. Their inhibitory effect was shown to be due to their action on gonococcal antigens and not on antibodies in the assay system. Some supernatants contained other inhibitors unaffected by STI; some of these were dialysable and others were not.

Sediments from the urine of patients with NSU or gonorrhoea were often strongly inhibitory, but treatment with STI annulled all but very slight inhibition. STI-treated sediments could, therefore, be used in an assay designed to detect gonococcal antigens.

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