DETECTION OF GONOCOCCAL ANTIGENS IN URINE BY RADIOIMMUNOASSAY


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Many recent attempts to provide a serological diagnosis of gonorrhoea aim to detect antibodies by sensitive methods such as radioimmunoassay (RIA) (Buchanan et al., 1973; Luoma, Cross and Rudbach, 1975; Oates et al., 1977), or immunofluorescence (O'Reilly, Welch and Rudbach, 1973; Welch and O'Reilly, 1973; Wilkinson, 1975; Gaafar, 1976; Gaafar and d'Arcangelis, 1976). In general, diagnostic methods based on detection of antibodies have two disadvantages: delay before detectable levels of antibody are produced, and the persistence of antibodies from a past infection which may lead to disease being diagnosed when it is absent.

Methods of detecting gonococci or gonococcal antigens do not have these drawbacks and culture methods, sometimes supplemented by fluorescent-antibody methods, are at present generally used to diagnose gonorrhoea. The investigation described here was designed to discover whether it is possible to detect gonococcal antigens in urine by a sensitive immunological method such as radioimmunoassay, in the hope that the test might be used for screening.

Experiments described below showed that a method of radioimmunoassay devised to detect antigens of Brucella abortus (Wilson, Thornley and Coombs, 1977) could be used in almost exactly the same form to measure gonococcal antigens in a buffer system.

However, application of this method to the detection of gonococcal antigens in urine samples was not straightforward. In preliminary experiments most of the free gonococcal antigen present and gonococci enclosed in pus cells were found in the urine sediment, even after very low-speed centrifugation, and

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a test for antigen in sediment had to be devised. In this, two main problems were encountered: non-specific binding of radioactively labelled IgG by substances present in urine, especially in the sediments, and inhibition of the assay by substances present in urine. Supernatants and sediments were clearly inhibitory, but the inhibition could not be studied quantitatively in the presence of non-specific binding. A study, described elsewhere (Thornley et al., 1979), was therefore made of the inhibition caused by supernatants; methods of preventing it were devised and applied in the experiments described here to neutralise inhibition when urine sediments were being tested for gonococcal antigen.

Thereafter, ways of reducing the non-specific binding of antibody by urine sediments were examined, and because some non-specific binding could not be removed, a test distinguishing between non-specific binding of antibody and its specific binding to gonococcal antigen was devised.

This paper describes: the RIA of gonococcal antigens in a buffer system; the specificity test devised to detect gonococcal antigens in urine sediments by RIA, and the prevention of inhibitory action; and the results obtained when this method was used to test the sediments of urine samples from male patients with gonorrhoea or non-specific urethritis, and urine sediments from a small number of female patients.

**MATERIALS AND METHODS**

_Gonococci_ were isolated from urethral, vaginal or cervical swabs, or urine samples, from the Genito-Medical Clinic, Addenbrooke's Hospital or from the Public Health Laboratory, Cambridge, by culture on chocolate (heated blood)-agar plates incubated for 24 or 48 h at 37°C in candle jars. Gonococci were identified by colony morphology, morphology after Gram staining, oxidase reaction, and fermentation tests. Rough counts of the number of gonococci in urine were made by spreading 0.1 ml of the sample over one third of a chocolate-agar plate, and streaking out for single colonies over the remainder. After incubation, the number of small translucent colonies composed of oxidase-positive gram-negative diplococci was recorded as: <100; 100-200; 200-500 or >500.

Preservation of cultures. Gonococci grown overnight on chocolate agar were suspended in a solution containing bovine serum albumen 5% (w/v) and monosodium glutamate 5% (w/v) (Greaves, 1960) and frozen in liquid nitrogen (Ward and Watt, 1971).

Antigen for production of antisera. Recently isolated strains of gonococci were grown overnight in Roux bottles in air with 10% CO₂ on either Columbia Agar Base (Oxoid) containing normal rabbit serum 10% (v/v), or Gc Agar Base (Oxoid) supplemented with BBL IsoVitaleX (Becton Dickinson) 1% (v/v), harvested, washed once with distilled water and stored as concentrated aqueous suspensions at -20°C.

Antigen for RIA was prepared by growing single strains on Gc agar with IsoVitaleX and washing once. A typical preparation was of dry weight 20 mg/ml, contained gonococci 3 × 10¹¹/ml estimated with Wellcome Opacity Tubes (Wellcome Reagents Ltd), and contained protein 17 mg/ml measured by the method of Lowry et al., (1951). Standard antigens consisted either of whole gonococci in suspension, or of gonococci disrupted ultrasonically by diluting a suspension 1 in 5 in phosphate-buffered saline (PBS) and exposing it for 3 min. to ultrasonic vibration of 20 Kc/s from a 100-watt ultrasonic disintegrator (MSE Scientific Instruments, Manor Royal, Crawley, Sussex). Whole and disrupted suspensions were stored at -20°C in small portions and thawed before use; material unused after thawing was discarded.

Preparation of antisera. The antiserum used in the radioimmunoassay was from a rabbit
DETECTION OF GONOCOCCAL ANTIGENS BY RIA (no. R5392) injected with six recently isolated strains of gonococci (nos. 1, 2, 3, 9, 10, and 12). The initial inoculation scheme, based on that of Apicella and Allen (1973), consisted of the subcutaneous injection of $5 \times 10^9$ gonococci in Freund's complete adjuvant on days 1 and 11 followed on days 22 and 24 by the intravenous injection of $5 \times 10^9$ gonococci without adjuvant. The rabbit was bled on day 31. Later series consisted of two intravenous injections, each of $5 \times 10^9$ gonococci, separated by a 3-day interval, the rabbit being bled 5 and 6 days after the last injection. This process was repeated 6 weeks and 3, 8, 10, and 14 months after the beginning of immunisation; antisera obtained after the sixth series of injections at 14 months was used. For inhibition experiments another antisera was used, from rabbit no. R5418 which had received injections at similar intervals but of material containing only four strains of gonococci.

Urine samples were from healthy male members of the laboratory and from patients attending the Genito-Medical Clinic of Addenbrooke's Hospital Cambridge, and the Praed Street Clinic, St Mary's Hospital, London by courtesy of Dr R. R. Willcox and Dr M. Jeffries.

At first, samples were collected only from men who had not received any recent antibiotic treatment, including men with gonorrhoea ("Gc urines") or non-specific urethritis ("NSU urines"); the latter were regarded as controls. Later, tests were made on samples from women attending the Genito-Medical Clinic for the first time. These included samples from women with gonorrhoea ("Gc urines") or with non-specific cervicitis, Trichomonas or Candida infections, and some in whom infection was not diagnosed ("non-Gc urines"). Urines were classed as "Gc urines" only when the initial diagnosis, based on clinical evidence and Gram-stained smears, was confirmed by culture.

After the collection of smears from the urethra of male patients, or from the urethra, cervix and vagina in females, the patients' bladder contents, up to a volume of 180 ml, were collected.

After urine samples had been plated out to detect gonococci as described above, the pH and content of pus and epithelial cells of the samples were measured; the 'pus cells' were predominantly neutrophil polymorphonuclear leucocytes. The samples were stored at 5°C until tested by RIA. This was done usually 2–7 days after collection, but occasionally after periods of up to 16 days. Repeated tests were made on samples stored for different times.

Preparation of urine sediments for RIA. Urine samples were warmed to 37°C for 30 min. to dissolve precipitates such as urates which would otherwise have sedimented and interfered with the test. When any precipitate such as phosphate remained and the urine was slightly alkaline, it was acidified to pH 5.5 with glacial acetic acid. After these treatments, precipitates persisted in very small amounts in only a few samples; some cellular material remained in samples containing many pus cells.

RIA was done on the sediment from 4-ml quantities of urine treated as described and prepared further by centrifugation at 2000 g for 10 min. and removal of the supernatant.

RIA materials and reagents. Radioactive sodium iodide (IMS-30) was obtained from the Radiochemical Centre, Amersham. Soybean trypsin inhibitor (STI) was the Type 11s, crude preparation T-9128 (Sigma Ltd, Poole, Dorset). Deoxyribonuclease (DNAase) was Sigma DH-100 and ribonuclease (RNAase) was Sigma R-4875. Assay buffer consisted of 500 ml of 0.9% (w/v) NaCl aqueous solution, 500 ml of 0.1 M sodium phosphate pH 7.5, 5 ml of 10% (w/v) sodium azide aqueous solution, bovine serum albumen 2 g, and Tween 20 5.5 g in early experiments and 11 g in later work.

The procedures used to prepare reagents were those of Wilson et al. (1977). IgG fractions of antisera were prepared by ion-exchange chromatography on QAE-Sepahex and portions of IgG were linked to micro-crystalline cellulose (E. Merck, Darmstadt) by the cyanogen bromide procedure of Wide (1969). Other portions of antibody-containing IgG were labelled by the chloramine-T method and purified on Sephadex G25 (Wilson et al., 1977). Typically, 10 μl of solution containing 10 μg of IgG were treated with 1 mCi of $^{125}$I; incorporation of label on to protein was usually about 70%.

The assays were carried out in small plastic tubes (LP3 tubes, Luckham Ltd, Burgess Hill). During incubation the tubes were rotated end-over-end at room temperature. Radioactivity was estimated in an LKB-Wallac automatic gamma counter.
Microbial product to be detected

Sample added to suspension of cellulose-antibody

Mix and incubate

Binding occurs

Spin and wash

Microbial products in pellet

$^{125}$I-labelled antibodies

Mix and incubate

Spin and wash

Pellet

Labelled antibody binds to microbial products

Bound radioactivity remains in pellet

FIG. 1.—Principle of the solid-phase labelled-antibody assay. Stage 1 (top row)—antigen capture. The sample or standard antigen is incubated with cellulose-antibody complex. Antigenic fragments are bound. Stage 2 (bottom row)—antigen detection. Radioactively labelled antibody in the second incubation binds to sites on the antigen other than those already bound. After washing, the amount of radioactivity in the pellet is related to the amount of antigen in the sample.
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**RIA procedure for gonococcal standard** (fig. 1 and table). **Stage 1**: the cellulose-antibody complex suspended in assay buffer was vigorously stirred while being dispensed with a Cornwall pipette into LP3 tubes. Each tube received 1 ml containing 0.125 mg (dry weight) of the complex and 20 μl of normal rabbit serum. Standard antigen, either whole or ultrasonically disrupted gonococci, was diluted in assay buffer, by either three-fold or ten-fold dilutions, and 100 μl samples of each dilution were added to the assay tubes. Tubes containing the cellulose-antibody but no antigen were treated in the same way to obtain background counts. All the tubes were incubated overnight, centrifuged for 3.5 min at 2000 g, the supernatants removed, and the pellets washed three times with 1.5 ml of assay buffer. **Stage 2**: the pellets were resuspended in 0.5 ml of assay buffer. Normal rabbit serum (20 μl) and 100 μl of 125I-labelled anti-gonococcal IgG with an activity of about 1000 counts/s were added. The tubes were incubated for 3 hours, centrifuged, the sediment washed three times with 1.5 ml of assay buffer and the radioactivity remaining in each tube counted.

**Modified RIA for testing urine sediments** (table). Each sample consisted of the sediment from 4 ml of urine, prepared as described above. The sediment was incubated for 1 hour with 0.4 ml of a solution containing STI 10 mg/ml and 20 μl of a solution containing DNAase 0.5 mg/ml and RNAase 2 mg/ml. **Stage 1**: for stage-1 incubation, 1 ml of the cellulose-antibody complex in assay buffer was added. To test for inhibition, some urine sediments were tested after the addition of gonococcal standard 10 μl at this stage ("S" test, table), and for comparison, gonococcal standards without urine sediment but with STI, DNAase and RNAase were included (table). Thereafter, incubation and washing were as described.

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<td><strong>Constituents of mixtures in each stage of the radioimmunoassay</strong></td>
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<td>urine-sediment test &quot;S&quot;</td>
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"P", "Q", and "S" tests: see Materials and methods. STI = soyabean trypsin inhibitor; Gc = gonococcus.
for stage 1 above. **Stage 2:** the labelling procedure was as described except that, for each urine, both "P" and "Q" tests were performed (table), to measure the specificity of the result. In the "P" test, 100 μl of a solution of normal rabbit IgG at a concentration of 720 μg/ml was added at the same time as the 125I-labelled antibody. In the "Q" test, 100 μl of non-radioactive anti-gonococcal IgG at a concentration of 720 μg/ml was added as well as the 125I-antibody. The "S" test and the gonococcal standard used for comparison were treated in the same way as the "P" test in stage 2.

**Calculation of results.** Initially, all tests were carried out in duplicate, and means of duplicate counts were used. Later, "P" and "Q" tests were carried out with five replicate samples from each urine sample. Results were calculated as % above background from the expression:

\[
\frac{x-b}{b} \times 100
\]

where \(x\) is the radioactivity retained by the tube containing sample or standard and \(b\) the radioactivity retained by an identical tube containing no antigen.

In the urine specificity test, results of "P" and "Q" tests were either expressed individually as % above background, or the difference between them was related to the background by the equation:

\[
P-Q = \frac{p-q}{b} \times 100
\]

where \(p\) and \(q\) are the means of counts of replicate tubes in each test.

Statistical evaluation of the significance of the difference between "P" and "Q", when five replicates were tested, was made by Student's \(t\) test.

**RESULTS**

**Specificity of gonococcal antiserum**

Although raised against only six strains of gonococci, the antiserum R5392 produced strong precipitin lines in gel-diffusion tests against ultrasonically disrupted preparations of 16 other strains of gonococci, and was therefore considered suitable for first trials of the RIA method. In preliminary trials with gel-diffusion tests and with RIA, serum R5392 cross-reacted strongly with three strains of *Neisseria meningitidis*, very weakly with *N. pharyngis*, and not at all with a non-pathogenic *Neisseria* sp. from urine nor with strains of other species commonly found in urine (*Escherichia coli*, *Proteus* sp., *Staphylococcus albus* and *Staphylococcus aureus*). In the experiments described, the serum was not absorbed with meningococci to remove cross reactions.

**RIA of standard preparations of gonococci**

A standard curve obtained with ultrasonically disintegrated gonococci is shown in fig. 2, in which results are plotted on a semi-logarithmic scale. The reproducibility of the assay was tested in five experiments on different dates, assaying duplicate samples of dilutions of standard antigen. The means and standard deviations of the ten results for each dilution are shown in fig. 2. Above the lowest values a linear curve was obtained.

The results are expressed in terms of nanograms of gonococcal protein in
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FIG. 2.—Standard curve for the assay of an ultrasonically-treated preparation of Gc strain no. 3. The results plotted are the mean of five separate assays on different dates, with duplicate samples; standard deviations of the mean are indicated.

Each 0.1 ml sample. The point representing 20 ng of protein, (log$_{10}$1.3) near the lower end of the scale, corresponds to material from $4 \times 10^5$ gonococci, judged by the opacity reading of the original standard suspension, and to 35 ng dry weight of gonococcal material. Because only a fraction of this would be measured as antigen, the sensitivity of the assay would probably be much greater with a pure antigen.

Inhibition of the RIA by unlabelled anti-gonococcal antibody

When non-radioactive anti-gonococcal IgG was added to the incubation mixture during the second stage of incubation at the same time as the $^{125}$I-labelled antibody, the radioactivity binding to standard antigen was very much reduced;
for example, fig. 3 shows the relation between the amount of antigen present, the amount of unlabelled antibody, and the degree of inhibition produced. It was concluded from this and similar experiments that the addition of 0.1 ml of an IgG preparation of R5418 anti-gonococcal antibody diluted ten-fold and containing protein 720 μl/ml completely inhibited the binding of radioactively labelled antibody to a portion of Gc standard containing 275 ng of protein (fig. 4), and reduced binding by two thirds when the antigen contained 16 500 ng of protein. These results formed the basis for the specificity test applied to urine sediments.
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Fig. 4.—Specificity tests on standard gonococcal antigen, containing 275 ng of protein, and urine sediments from male patients. When the radioactivity bound in both "P" and "Q" tests is equal or nearly equal, the binding is non-specific (urine nos. 385 and 369). Specific binding to gonococcal antigens is inhibited in "Q" tests by non-radioactive anti-gonococcal IgG, and the difference "P—Q" is a measure of gonococcal antigens, as shown by the Gc standard and urines 377 and 426.
Specificity tests on urine sediments

Each sediment was tested after the addition of unlabelled anti-gonococcal IgG ("Q" test) and also after the addition of an equal amount of IgG from a pool of normal rabbit serum ("P" test), and the two results were compared. The binding of radioactivity in the "P" test was considered to be due to gonococcal antigen only if binding was lower in the "Q" test; the value "P - Q" was then used as a measure of the amount of gonococcal antigen present.

Non-specific binding of radioactively labelled immunoglobulin

When the radioactivity bound was the same, or very nearly the same, in "P" and "Q" tests, this was considered to be due to non-specific binding of the labelled immunoglobulin to some component of the sediment. Non-specific binding was very low in 10 samples of urine from healthy men, "P" and "Q" values being less than 53% above background, and it was very low or moderately low in 27 NSU urines, for which two typical results are shown in fig. 4. The NSU urines contained from less than 10 to 820 pus cells/mm³, but, because non-specific binding was not related to a sample's content of pus cells, it was concluded that some other component must be responsible for non-specific binding.

When Gc urine sediments were being tested, non-specific binding was estimated from the results of the "Q" test. These urines contained from less than 10 to 4500 pus cells/mm³, and those with up to 500 pus cells/mm³ resembled the NSU urines in the amount of their non-specific binding. When pus cell counts were higher than this, much greater non-specific binding was usually, but not always, found. For instance (fig. 4), sample no. 377 containing 990 pus cells/mm³ did not bind radioactivity non-specifically, while sample no. 426, containing 840 pus cells/mm³ gave moderately high binding.

Specific binding of radioactive anti-gonococcal antibody

This was measured by the difference "P - Q" in experiments with duplicate samples. When samples from 10 healthy men were tested, this difference varied from -6% to +47% above background, with a mean of 19%, while in tests on 27 NSU urines (fig. 5) "P - Q" values were from -46% to +43%, with a mean of 9% above background.

Results of tests on duplicate samples of 31 Gc urine sediments from male patients are compared with those for NSU urines in fig. 5. Values ranged from -15% to +607%, with a mean of 176% above background. Clearly, many of these results were much higher than any found with NSU urines and therefore indicate the detection of gonococcal antigen. For a full-scale study and if it were decided to use duplicate samples, the level at which "P - Q" results should be regarded as positive would be determined statistically with many more samples. We assumed, arbitrarily, that NSU samples were unlikely to
give "P-Q" results greater than 60% above background, and on this basis 23 (74%) of 31 Gc urines tested gave positive results for gonococcal antigens. By testing five replicate samples in "P" and "Q" tests it was possible to evaluate with a t test the significance of the difference "P-Q" for each urine sample. The results obtained by testing 11 Gc urines from men were evaluated in this way. The difference "P-Q" was significant at the 0.1% level of probability for eight, with "P-Q" values ranging from 44% to 325% above background. The differences were not significant at the 5% level for three samples with "P-Q" values from 8% to 17% above background. Five NSU urines from men gave "P-Q" values from -15% to +16% above background and were not significant at the 5% level. Thus, the difference between positive and negative results was very clear-cut, with no intermediate values of probability. When all results for men with gonorrhoea, tested with either two
or five replicate samples, were combined, 31 (74%) of 42 tested gave positive results for gonococcal antigens.

However, when five replicates of samples from a small number of women patients were tested, 10 of 14 Gc urine sediments gave positive results significant at the 2.5% level, and three of 18 non-Gc urines gave positive results at the same level of significance.

**Effect of storage**

Urine from male patients was stored at 5°C, and each urine was tested twice, the first test being 2–7 days after collection, and the second at various times up to 27 days after collection. At each test, duplicate sediments were prepared for "P" and for "Q" determinations. "P–Q" values for 11 NSU urines were low at the first test and remained low after 14 or 23 days storage. Values for 13 Gc urines either increased, decreased, or remained constant when results for the first and second tests on the same urine were compared, but only one sample, initially positive with a "P–Q" value of 62%, became negative after storage for 14 days. Six samples which were positive at the first test remained so after storage for 20–27 days.

**Relation between "P–Q" values and other properties of Gc-urine sediments**

From fig. 6, which relates to the Gc urine results shown in fig. 5, it can be seen that urines that failed to yield viable gonococci from a sample of 0.1 ml were negative or gave borderline results in the "P–Q" test and also contained few pus cells. With increasing numbers of colonies of gonococci, the "P–Q" values tended to increase but, within each category of colony numbers, there was a wide range in "P–Q" values. This may reflect differences between strains of gonococci, in that some may react better with the antiserum used. However, it is clear that high "P–Q" values tended to be associated with high pus-cell counts. This suggests that the assay detected gonococci originally present within pus cells in the sample. Whether these were viable or not at the time of plating, the colony count would not give an accurate estimate of numbers, because only one colony would be likely to be formed from one pus cell.

The appearance of the urine sediment by phase-contrast microscopy after the first, overnight, incubation, suggested that most pus cells were lysed by being rotated in detergent-containing buffer. A few cell "ghosts" remained, but had apparently lost their contents, supporting the idea that the assay detected gonococci that were originally present within pus cells.

**DISCUSSION**

That the solid-phase RIA method used here can be a very sensitive way of detecting bacterial antigens, even without affinity purification of the antibody, has been shown by results with brucella antigens (Wilson et al., 1977). The assay for gonococcal antigens appeared to be less sensitive, possibly because the standard antigen was derived from whole gonococci, only a fraction of
which is measured as antigen in the test, whereas the brucella test was done with an extract enriched in surface antigens.

The method was primarily intended for use with soluble antigens, and it was hoped at the beginning of this study that enough soluble surface antigens of the gonococcus might be liberated, and therefore assayable, in urine supernatants. However, it was clear from early results that much more gonococcal
material was found in the urine sediment, possibly associated with pus cells. Tests on urine sediments were complicated by the presence of material that retained radioactively labelled antibody non-specifically. Nevertheless, it was possibly to overcome this difficulty by carrying out a specificity test on each sample. Some heat-resistant substances in urine which inhibit the RIA of gonococcal antigens have been shown by Thornley et al. (1979) to be present only in the supernatants, and would therefore not affect the test on urine sediments. The inhibitory action of proteolytic enzymes was due to their effect on the gonococcal antigens; it was present in supernatants and sediments, and could be completely prevented by the addition of soybean trypsin inhibitor.

Our results show that gonococcal antigens in urine can be detected by radioimmunoassay, 31 (74%) of 42 samples from men with gonorrhoea, and 10 of 14 samples from women with gonorrhoea being positive. In these first experiments with this method, antibody to whole gonococci was used and it was not affinity-purified to isolate antigonococcal antibodies from the IgG. The use of purified antigens and affinity purification of antibody should improve greatly the sensitivity of the assay, which should then be able to detect nearly all men and a useful proportion of women with gonorrhoea. Although gonococci are present in smaller numbers in urine from women patients than in that of men, Chapel and Smeltzer (1975) detected viable gonococci by culture from urine sediments from 72% of female patients.

Much of the gonococcal antigen in urine is probably associated with gonococci killed by phagocytic action or in other ways. The radioimmunoassay gave the same result whether the gonococci were alive or not, and was little affected by prolonged storage of urine samples at 5°C. It also seems likely that gonococci originally enclosed within pus cells are detected by the assay. For these reasons radioimmunoassay has advantages compared with culture methods, because delay in transit of specimens, unsuitable temperatures during transit, or treatment of the patient with antibiotics may all cause gonococci to die and lead to failure to detect them by culture.

**Summary**

A method of detecting gonococcal antigens by solid-phase radioimmunoassay with radioactively labelled antibody is described. A specificity test has been developed that enables this method to be used to detect gonococcal antigens in urine sediments. When sediments from samples of urine from male patients with gonorrhoea were tested, 31 (74%) of 42 gave positive results, clearly distinguishing them from sediments from urine samples from men with non-specific urethritis, none of which was positive. Ten of 14 urine sediments from urine samples from women with gonorrhoea gave positive results, as did 3 of 18 sediments from urine samples from women patients without gonorrhoea.

These experiments demonstrate that gonococcal antigens can be detected in urine by radioimmunoassay; the method could be useful in diagnosis if, after refinement, its sensitivity and specificity were to be increased.
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