Antigens of *Mycobacterium leprae* and anti-*M. leprae* antibodies in the urine of leprosy patients

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Summary Forty-six newly diagnosed untreated leprosy patients and 11 control persons were examined for acid-fast bacilli, *Mycobacterium leprae* antigen(s) and anti-*M. leprae* antibody-like activity in concentrated urine samples. No acid-fast bacilli were found. Two of 23 paucibacillary and 11 of 23 multibacillary patients had detectable antigen(s) in the urine. The antigen(s) was/were absorbed by anti-BCG in 7 out of 8 examined samples. A significant correlation between maximal bacteriological index and antigen concentration was found. Extensive differences in the anti-*M. leprae* antibody-like activity were seen within the group of control persons and the different groups of patients throughout the spectrum of leprosy. The results of examinations of the antibody-like activity after absorptions with Cowan I staphylococci, Sepharose–anti-human IgG, heat treatment and assays for total IgG, IgA and IgM gave good evidence that most of the activity was due to anti-*M. leprae* antibodies of the IgG class.

Introduction

Clinical leprosy is recognized by the clinical signs and symptoms, the documentation of acid-fast bacilli in slit-skin smears and histopathologically. Sometimes *in vivo* or *in vitro* immunological tests can be helpful in the diagnosis of clinical leprosy.

The diagnosis of subclinical leprosy (defined as infection without clinical signs and symptoms at any time after infection) and preclinical leprosy (defined as the

stage of infection before the clinical signs and symptoms have developed; this corresponds to the incubation period) can be done by documenting the bacilli and/or the histopathological changes these may cause. This is, however, difficult when the location of the infection is unknown. The use of immunological methods to measure these responses to the infection offers one possibility for diagnosis of sub- and preclinical leprosy.

Tests for cell-mediated immunity include, for example, lepromin test lymphocyte transformation test and leukocyte migration inhibition test.^{1,2} The problems with these tests are, however, that (a) persons who will develop lepromatous leprosy probably have a bad response to *Mycobacterium leprae* antigens already in the preclinical period; (b) positive results are difficult to interpret with respect to the time that an individual was infected, unless prospective studies are conducted with repeated tests; (c) subclinical infections that have been spontaneously cured cannot be differentiated from preclinical infections; and (d) the tests lack specificity and cross-react with other mycobacteria, for example *M. tuberculosis* and BCG.³

Tests for anti-*M. leprae* antibodies have also been tried in the diagnosis of sub- and preclinical leprosy.⁴⁻⁹ This approach overcomes some of the disadvantages listed above. For example, most lepromatous patients develop anti-*M. leprae* antibodies^{4,10} in spite of their low or lacking cell-mediated immune response to *M. leprae*. The antibody tests have also been refined to decrease the cross-reactivity with other mycobacterial infections.^{4,6} The drawbacks mentioned under (b) and (c) above are, however, still attached to these tests.

Another diagnostic method would be to document the presence of *M. leprae* antigens. This has been done in tissue biopsies from patients with diagnosed leprosy.¹¹ In other infectious diseases like bacterial meningitis,¹² pneumococcal pneumonia¹³ and *Schistosoma mansoni* infections¹⁴ microbial antigens have been found in body fluids like blood and urine. If *Mycobacterium leprae* antigens could be found in body fluids like urine it may offer a better method for diagnosing sub- and preclinical leprosy. The theoretical advantages would be that detection of antigen ought to be closely correlated to active infection and that urine is a much easier sample to collect than blood, skin smears and biopsies, especially in epidemiological field surveys.

The present study was initiated due to the theoretical advantages of antigen detection discussed above with the primary aim to see if *M. leprae* antigens were to be found in urine or not. Since untreated newly diagnosed patients with clinical leprosy were thought to be the ones most likely to have detectable levels of *M. leprae* antigens, the study was focused on this group. It was also realized that the anti-*M. leprae* antibody-like activity, found in many urine samples in pilot studies that were conducted by us, needed to be further explored. The results of these studies are also included in the present paper.

Material and Methods

PATIENTS

Forty-six untreated leprosy patients attending the out-patient clinic of ALERT between March and July 1982 were included. Their ages ranged from 9 to 51 years (mean 28 years) and the history of clinical signs and symptoms varied between 3 months and 9 years (mean 2 years and 9 months). The diagnosis was based on the clinical findings, the results of examining slit-skin smears for acid-fast bacilli (Ziehl–Neelsen staining) and in most cases by histopathological examination of skin biopsies. The classification was performed according to the Ridley–Jopling scale.¹⁵ Reversal reactions were seen in 2 borderline tuberculoid (BT) and 2 borderline lepromatous (BL) patients. None of the patients was suffering from erythema nodosum leprosum.

Four healthy Ethiopian laboratory technicians, who have been working extensively with *Mycobacterium leprae* for several years, and 7 patients admitted to the ALERT hospital without leprosy but with the diagnosis of pemphigus vulgaris, diffuse cutaneous leishmaniasis, cataract, glaucoma and keratitis due to onchocerciasis were included as controls.

URINARY SPECIMENS

One hundred to three hundred millilitres of urine was collected from each patient before start of antileprosy treatment. The urine was stored at +4°C for 1–3 days after addition of sodium azide (final concentration 1 mg/ml). Tests for blood, glucose, protein and nitrite was carried out using Ames N-multistix. The urine was centrifuged at 17,000 *g* for 20 minutes. The sediment was examined for cells, cylinders and bacteria after staining with crystal violet (0.1%) and safranin (0.2%). Staining for acid-fast bacilli was performed with the Ziehl–Neelsen technique and by the fluorochrome auramine.¹⁶ At least 100 high-power fields were examined in each slide. The supernatant was concentrated about 10 times in an ultrafiltration cell (Amicon model 402) with a Diaflo ultrafilter YM 10 (cut-off 10,000 MW). The source of pressure (4.5 bar) for operating the cell was dried nitrogen gas. Double distilled water was added to reach the original volume which was again reduced to one-tenth of the original in the cell. This volume was lyophilized. The dry powder was dissolved in 'RIA-buffer' (0.01 M phosphate-buffered saline pH 7.4 = PBS, with 0.2% bovine serum albumin—Sigma Chemical Co, USA—and 0.2 mg NaN₃/ml) to a volume 1:100 the original, and stored at –20°C until assayed.

The concentrations of total IgG, IgM and IgA in the concentrated urine samples were measured by the Mancini technique (standard solutions from Behringwerke AG).

KIDNEY FUNCTION

This was evaluated in each patient by the history and general physical examination including blood pressure, the serum creatinine level (Créatinine-kit Bio Mérieux) and the urine tests noted above.

RADIOLABELLING OF ARMADILLO *M. LEPRAE* ANTIGENS

Freeze-dried armadillo *M. leprae* batch CD12, prepared according to Protocol 1/79, was provided by IMMLEP component of UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. The bacilli were sonicated twice in 0.15 M NaCl, first with 50 W for 5 minutes and a second time, after storage of the suspension over night at +4°C, with 100 W for 5 minutes. The sonicated suspension was centrifuged at 20,000 *g* for 20 minutes and the supernatant concentrated to 0.3 mg protein/ml. This preparation was named the armadillo *M. leprae* sonicate (AMIS) and was radiolabelled ¹²⁵I by the electrolytic iodination method described by Harboe and Fölling.¹⁷

In short 400 µl of AMIS was mixed with 40 µl 5 × 10⁻⁵ M NaI, 160 µl distilled water and 1 m Ci ¹²⁵I (supplied as sodium iodide in dilute sodium hydroxide by Amersham International, England) in a stirred platinum beaker. A current of 15–25 mA was run for 15–25 minutes between the beaker and a platinum electrode dipped into the AMIS. 200 µl of 1 × 10⁻² M NaI was added and the solution dialysed extensively against RIA-buffer. After centrifugation at 20,000 *g* for 20 minutes the supernatant was tested for antigenicity and stored at +4°C. The method of radiolabelling has been shown to give a strong labelling of *M. leprae* antigen 7.¹⁸

RADIOIMMUNOASSAY (RIA) FOR DETECTING ANTI-*M. LEPRAE* ANTIBODY-LIKE ACTIVITY IN CONCENTRATED URINE SAMPLES

Samples were run in duplicate. Each tube contained 50 µl of concentrated urine, that had been cleared of sedimenting particles by centrifugation at 2300 *g* for 10 minutes after thawing; 50 µl of RIA-buffer; and 50 µl of (¹²⁵I)-AMIS diluted to give 125,000 counts in 400 seconds. The mixture was incubated for 30 minutes at room temperature (Rt). Controls without urine and (¹²⁵I)-AMIS were run at the same time. For reference values of anti-*M. leprae* antibody-like activity different concentrations of a high titre patient anti-*M. leprae* serum (no. 625 in ref. 18) was also run in the assay.

(¹²⁵I)-AMIS bound to antibodies, or antibody-like substances, was separated from free AMIS by formalinized staphylococci (1 ml of a 1% suspension) strain Cowan I (Pharmacia Diagnostics AB, Sweden). The staphylococci (CI) were pelleted by centrifugation at 2300 *g* for 20 minutes, the supernatant aspirated and the pellet counted in a LKB-Wallac 1270 rackgamma II gamma counter for 400 seconds.

In order to get some idea of the nature of anti-*M. leprae* antibody-like activity, the concentrated urine samples were treated in different ways before the RIA. Samples were absorbed with different amounts of CI; heat treated in boiling water (+92.5°C at the altitude of Addis Ababa) for 5 minutes; absorbed with different amounts of Sepharose 4B (Pharmacia Fine Chemicals AB)-conjugated rabbit anti-human IgG (DAKO-immunoglobulins a/s, lot 110B).

INHIBITION RIA FOR DETECTING *M. LEPRAE* ANTIGENS IN CONCENTRATED URINE SAMPLES

Samples were run in duplicate. Each tube contained 50 µl of a 10⁻³ dilution of patient anti-*M. leprae* antiserum 625. In addition to being present in a high titre the antibodies had previously been shown¹⁸ to be blocked by the mycobacterial polysaccharides arabinogalactan (AG) and arabinomannan. Fifty microlitres of concentrated urine was added after inactivation of the antibody-like activity by absorbing 100 µl of urine with 20 mg wet weight CI for 5 minutes at Rt and heat treatment. Fifty microlitres of properly diluted (¹²⁵I)-AMIS was added and the further steps in the assay were run as for antibody-like activity. Controls without anti-serum 625, urine and (¹²⁵I)-AMIS were included. Different concentrations of a crude AG preparation from *M. tuberculosis*, prepared as previously described,¹⁸ were assayed at the same time to give reference values to inhibiting substances in the urine samples.

In order to see if inhibition in the assay was due to the presence of mycobacterial antigens in the urine, absorptions were made before the assay with coagglutination reagents made by mixing 100 µl of anti-BCG antiserum (DAKO, lot 068C) or 100 µl of normal rabbit serum (NRS, DAKO lot 109B) with 1 ml of a 10% suspension of CI.^{19,20} The mixture was kept at Rt for 5 minutes, centrifuged at 2300 g for 10 minutes, the supernatant aspirated and the pellet resuspended in PBS with 1% tween 20.

Results

KIDNEY FUNCTION IN NEWLY DIAGNOSED UNTREATED LEPROSY PATIENTS

All the 46 patients included in the study except 1 showed normal renal function (as evaluated by the clinical examination, serum-creatinine, the negative findings of blood and protein in urine and normal urine sediment). One patient with LL for 3 years without inflammatory complications had microscopic haematuria but no proteinuria. No patient had evidence of urinary tract infection as evaluated by the history, physical examination, nitrite test and urine sediment. No case of glucosuria was found.

URINE SEDIMENT EXAMINED FOR ACID-FAST BACILLI

Acid-fast bacilli were not seen in the stained urinary sediments from any of the 46 patients.

ANTI-*MYCOBACTERIUM LEPRAE* ANTIBODY-LIKE ACTIVITY IN CONCENTRATED URINE SAMPLES

Figure 1 shows that almost all the urine samples showed higher antibody-like activity than a 10^{-5} dilution of the high titre patient serum 625. Some urine

Figure 1. Results of RIA for anti-*M. leprae* antibody-like activity expressed as counts per minute (CPM) for individual concentrated urine samples in different groups of leprosy patients and control persons (CP). The horizontal lines represent the CPM with different dilutions of the high titre anti-*M. leprae* patient serum 625 (see 'Material and methods').

samples had very high antibody-like activity. In order to further explore the nature of this activity additional tests were performed and the following results were observed:

1 Binding to the gamma-vial wall of a urine-(^{125}I)-AMIS complex was ruled out by resuspending counted pellets, repelleting them in new gamma-vials and recounting the pellet and the empty vial. Only background values were obtained from the empty vial.

2 Formation of precipitates between some urine components and (125 I)-AMIS that pelleted together with CI could not be ruled out.

3 Direct binding of (125 I)-AMIS to CI was not found since the controls with just these two components gave low counts (300 CPM in Figure 1). In addition absorptions of (125 I)-AMIS with CI (100 μ l with 20 mg) did not affect the results of the assay for antibody-like activity in the urine.

Figure 2. Results of RIA for anti-*M. leprae* antibody-like activity expressed as CPM for the 5 concentrated urine samples from leprosy patients with the highest anti-*M. leprae* antibody-like activity after (a) absorptions with different amounts of Cowan I (CI) staphylococci per 100 μ l of urine, and (b) absorptions with different amounts of Sepharose-anti-human IgG per 100 μ l of urine.

4 Binding of (125 I)-AMIS to CI by anti-mycobacterial antibodies or by a non-specific component in urine was documented by absorbing urine samples before the assay with CI. Twenty milligrams CI wet weight per 100 μ l of urine absorbed all anti-*M. leprae* antibody-like activity completely except in the 5 samples with the highest antibody-like activity. The results obtained by absorptions with 20, 40, and 80 mg CI (wet weight) per 100 μ l urine is shown in

Figure 2a. In 4 of the samples all activity was absorbed by 80 mg CI while the activity was substantially reduced in the fifth sample.

In order to see if antibodies or non-specific components were responsible for the coupling of (125 I)-AMIS to CI the concentrated urine samples were absorbed with Sepharose 4B-anti-human IgG before the assay. The urine samples were also examined for total immunoglobulin G, M and A.

The 5 concentrated urine samples with the highest anti-*M. leprae* antibody-like activity, the same as in Figure 2a, were absorbed by different amounts of Sepharose-anti-IgG (Figure 2b). In 4 of the samples the antibody activity was markedly reduced while the fifth showed a slight decrease in activity after absorption with 80 mg Sepharose-anti-IgG (wet weight). The dilution of a sample by the absorption procedure came to 4–9% so the results are not explained by dilution of the samples.

Figure 3. Results of examination of concentrated urine samples for IgG. Open bars represent leprosy patients and hatched control persons. n = number of persons.

All the samples from patients as well as control subjects contained IgG (Figure 3), or fragments of IgG, as shown by a Mancini test. There was no correlation between total IgG and anti-*M. leprae* antibody-like activity. IgA was found in trace amounts (less than 10 mg/100 ml) in 47 concentrated urine samples. In 7 samples no IgA was documented and in 3 the concentrations were 19, 73 and 106 mg/100 ml respectively. IgM was found in trace amounts (less than 30 mg/100 ml) in 6 samples while the remaining were negative.

Heat treatment completely destroyed all the anti-*M. leprae* antibody-like activity.

M. LEPRAE ANTIGENS IN CONCENTRATED URINE SAMPLES

Figure 4 shows the results of inhibition RIA for detection of *M. leprae* antigens. The relation of a urine sample count to the mean buffer value (1003 CPM) is expressed as a percentage, after subtraction of the mean background value (369 CPM) from each of them. The inhibition by different concentrations of AG is also indicated in Figure 4. Using Student's *t*-test (one tail) after square root

Figure 4. Results of inhibition RIA for *M. leprae* antigen in concentrated urine samples from different groups of leprosy patients and control persons (CP). The radioactivity in a sample is expressed as a ratio (%) calculated as follows:

$$100 \times \frac{\text{CPM for sample} - \text{CPM of background}}{\text{CPM for buffer} - \text{CPM of background}}$$

The horizontal lines represent the ratio for different concentrations of arabinogalactan (AG).

transformation of the results for the control persons only 1% of these could be calculated to get values lower than 79.6% by chance. The same level of inhibition was seen by 10^{-5} mg AG/ml.

It can be seen that 2 out of 18 (11%) urine samples from BT patients, 6 out of 15 (40%) samples from BL patients and 5 out of 8 (63%) samples from LL patients showed a markedly higher inhibition than the inhibition by 10^{-5} mg AG/ml.

Three of the urine samples from BL patients and 5 from LL patients with the

highest inhibition were selected for absorptions with CI-anti-BCG and CI-NRS co-agglutination reagents in order to study if anti-BCG could absorb the AG-like inhibition.

It was found that in 7 of the 8 urine samples absorption with CI-anti-BCG gave a reduced inhibition as compared to absorption with CI-NRS. The reduced inhibition corresponded to an absorption of 1.6×10^{-4} to 3.4×10^{-3} mg AG/ml urine (mean 8.0×10^{-4}) by the CI-anti-BCG reagent. This absorption study thus suggests that most of the urine samples with a high inhibition contained mycobacterial antigen(s) that could be absorbed with a CI-anti-BCG reagent.

Heat treatment of dilutions of AG and concentrated urine samples did not alter the inhibitory effect in the present assay.

Figure 5. Correlation of maximal bacteriological index (BI max), as seen in slit-skin smears from 6 different sites, with the inhibition RIA for *M. leprae* antigen in concentrated urine samples from BL and LL patients. The ratio (%) is calculated as presented in Figure 4. For interpretation of BI numbers see.²¹ The equation for the regression line is $Y = 109.9 - 7.64 \cdot X$.

The correlation between inhibitory activity and the highest bacteriological index (BI) found in 1 site out of 6 in the BL and LL patients (Figure 5) was significant (correlation coefficient 0.71, $P < 0.1\%$, 21 degrees of freedom) for a linear average relationship between the 2 parameters.

Discussion

The approach of antigen detection for the diagnosis of an infectious agent has definite advantages over measurements of antibodies or cell-mediated immune

response. The presence of antigens is, for example, more closely related to active infection. In leprosy antigens may, however, also be found in the body for a long time after the bacilli are dead as suggested by the slit-skin examinations of treated patients.²²

The antigen detection is a theoretically interesting possibility for the diagnosis of leprosy before clinical signs and/or symptoms develop (= preclinically). This would of course be of great value to the patient, who could get treatment earlier, perhaps without development of clinical disease, as well as for the society when contagious multibacillary cases could be diagnosed and treated earlier than at present.

Other interesting aspects of antigen detection are that quantitative measurements of antigen excretion in urine, over for example 24 hours, may be an additional way to evaluate the load of *Mycobacterium leprae* in a patient and also a way to follow the effect of treatment.

The use of urine for antigen detection is a favourable approach since the sampling is non-traumatic and urine is easily passed without hesitation by persons in most societies. These factors may prove favourable in epidemiological studies of leprosy.

The present study was conducted to see if *M. leprae* antigen(s) could be detected in urine in view of the possible advantages mentioned above. It was soon found out that the concentrated urine samples had an anti-*M. leprae* antibody-like activity that destroyed the antigen detecting assay. It is well known that Ig, mainly class G, and fragments of Ig are found in the urine from normal persons,²³ patients with non-renal infectious diseases²⁴ including leprosy.²⁵ This was also found in our patient and control urine samples which all contained IgG or at least antigenic fragments of IgG that gave precipitation with the anti-IgG antiserum used. It is also well documented that IgG (subgroup 1, 2 and 4) is the main class of human Ig that binds to CI²⁶ and therefore it is likely that urine IgG with antibody specificity to mycobacterial antigen(s) is responsible for the interference seen in the antigen assay.

The interfering activity was inactivated by absorption of the urine by CI-Sepharose anti-human IgG and by heat treatment. These results are expected if IgG is responsible for the interference.

Whether the anti-*M. leprae* antibodies found in urine can be of any diagnostic/epidemiological use or not is an open question.

In multibacillary leprosy patients acid-fast bacilli can be found in peripheral blood.²⁷ In spite of this sign of systemic spread of the bacillus there are few reports of acid-fast bacilli and histopathological patterns corresponding to leprosy lesions in kidneys.²⁸⁻³³ The reported finding of acid-fast bacilli in urine from a high proportion of leprosy patients, even after long periods of treatment³⁴ has not been confirmed and finds no support in the present study.

Renal damage has been reported in a high percentage of leprosy patients. The patients examined in these reports have mostly been treated for longer periods

before their kidneys were biopsied and have had complicating inflammatory reactions. They thus differ from our material from newly diagnosed and untreated patients who all, except 4, had not experienced any reactions. With the methods available only 1 patient had evidence of kidney damage by having an isolated haematuria. Causes for the haematuria other than renal disease were, however, not fully evaluated in this case.

The presence in concentrated urine of substance(s) inhibiting the binding of (125 I)-AMIS to a reference serum with anti-*M. leprae* antibodies was documented in 11 of 23 multibacillary cases and in 2 of 23 paucibacillary patients. Support for the idea that the inhibition was due to mycobacterial antigen(s) was found by the markedly reduced inhibition seen after absorption of 7 out of 8 urine samples with CI-anti-BCG and by the significant correlation of the BI to the inhibition. The nature of this/these antigen(s) has not been revealed so far but work is in progress to clarify this by immunological and biochemical techniques.

A significant linear average relationship between inhibitory activity and the highest BI was found (Figure 5). Since the BI scale is logarithmic the inhibition is not directly proportional to the number of bacilli found in a slit-skin smear but to the logarithm of this number. The same linear relation was found for the inhibition and the logarithm of different concentrations of AG. This can to some extent be seen from results presented in Figure 4 where 10^{-5} , 10^{-4} and 10^{-3} mg of AG per millilitre gave ratios of 79%, 63% and 39% respectively. The inhibition RIA thus seems to give a linear relationship of the inhibition to the logarithm of the antigen concentration within the interval of concentrations here studied.

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