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References

1 Tanner, J M, *et al*, *Archives of Disease in Childhood*, 1971, **46**, 745.
2 Wise, P H, *et al*, *Archives of Disease in Childhood*, 1975, **50**, 210.
3 Saenger, P, *et al*, *Journal of Pediatrics*, 1974, **84**, 137.
4 Preece, M A, and Tanner, J M, *Journal of Pediatrics*, 1977, **89**, 334.
5 Preece, M A, 1977, personal communication.
6 Tanner, J M, Whitehouse, R H, and Takaishi, M, *Archives of Disease in Childhood*, 1966, **41**, 454.
7 Roth, J, *et al*, *Metabolism*, 1963, **12**, 577.
8 Hunter, W M, *et al*, *Lancet*, 1967, **2**, 1271.
9 Buckler, J M H, *Acta Endocrinologica*, 1972, **69**, 219.
10 Lacey, K A, Hewison, A, and Parkin, J M, *Archives of Disease in Childhood*, 1973, **48**, 508.
11 Hunter, W M, in *Methods of Hormone Analysis*, ed H Breuer, D Hamel, and H L Kruskemper. New York, J Wiley and Sons, 1975.
12 Wedge, P, and Prosser, H, *Born to Fail?* London, Arrow Books, 1973.
13 Tanner, J M, Goldstein, H, and Whitehouse, R H, *Archives of Disease in Childhood*, 1970, **45**, 755.
14 Tanner, J M, Whitehouse, R H, and Healey, M J R, *A New System for estimating Skeletal Maturity from the Hand and Wrist with Standards derived from the Study of 2600 Healthy British Children*, parts I and II. Paris, Centre International de l'Enfance, 1962.
15 *Medical Journal of Australia*, 1974, **1**, 909.
16 Penny, R, Blizzard, R M, and Davis, W T, *Journal of Clinical Endocrinology and Metabolism*, 1969, **29**, 1499.
17 Poskitt, E M E, and Rayner, P H W, *Archives of Disease in Childhood*, 1974, **49**, 55.
18 Rimoin, D L, Merimee, T J, and McKusick, V A, *Science*, 1966, **152**, 1635.
19 Rona, R J, and Tanner, J M, *Archives of Disease in Childhood*, 1977, **52**, 197.
20 Parkin, J M, *Archives of Disease in Childhood*, 1974, **49**, 904.
21 Tanner, J M, *Health Trends*, 1975, **7**, 61.

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Antibodies against BCG antigen 60 in mycobacterial infection

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Summary

A sensitive specific radioimmunoassay was developed to measure antibodies against BCG antigen 60, a prominent antigenic component of BCG bacilli which cross-reacts with similar components in many mycobacterial species including Mycobacterium leprae and M tuberculosis. A lepromatous serum pool had anti-BCG-60 activity with a titre of 10^5 and the tuberculoid pool a titre of 10^4. Testing of individual sera showed striking variations within groups of patients with lepromatous and tuberculoid leprosy. In five of the 20 tuberculoid leprosy sera the anti-BCG-60 activity was above the median for the lepromatous group. The current view that antibody formation against mycobacterial antigens is very low in tuberculoid leprosy thus no longer appears to be tenable. Sera from eight patients with active pulmonary tuberculosis also showed a striking variation in anti-BCG-60 content, and the median value of this group was even higher than in those with lepromatous leprosy.

Introduction

Leprosy is an example of a "spectral" disease—that is, one that shows considerable variations in clinical course.[1-5] The current view is that polar lepromatous leprosy is characterised by lack of cell-mediated immunity towards *Mycobacterium leprae*, apparent uninhibited growth of the bacilli, and a high serum content of antibodies against various mycobacterial antigens. In the highly resistant form, tuberculoid leprosy, the reverse is the case: cellular immunity and hypersensitivity are well developed whereas antibodies to mycobacterial antigens occur infrequently and in low concentration.

Crossed immunoelectrophoresis (CIE) has shown that several mycobacterial species contain more than 40 different antigenic components.[6-9] To gain more insight into the immunology of leprosy and other mycobacterial diseases there is a great need for additional information on the immune response of the host against individual antigenic components of the mycobacteria.

Antigen 60 is a prominent antigenic component of BCG bacilli which cross-reacts with similar components in many mycobacterial species, including *M leprae* and *M tuberculosis*. We have produced monospecific antibodies against this antigen and have developed a specific radioimmunoassay (RIA) for measuring anti-BCG-60 antibodies in rabbit sera.[10] We report here our studies of anti-BCG-60 antibodies in sera from patients with lepromatous and tuberculoid leprosy and from several groups of controls, including patients with active pulmonary tuberculosis. The results obtained represent a challenge to the simplistic immunological model of leprosy outlined above.

Patients and methods

Purification and labelling of BCG antigen 60 by electrolytic iodination has been described in detail elsewhere.[10] The preparation was tested as shown in fig 1. A mixture of 5 μl of concentrated culture fluid obtained after cultivating BCG bacilli for four weeks on Sauton's medium and 5 μl of labelled antigen 60 were placed in the circular well and tested by CIE. The top gel contained 200 μl of concentrated anti-BCG immunoglobulin (Dakopatts A/S, Copenhagen; lot No 115). The plates were washed, pressed, stained with Coommassie brilliant blue, and dried as described elsewhere.[11] For autoradiography an *x*-ray film (Kodak RP 14 safety film) was applied directly to the stained glass plate with an exposure time of 18 days. The left part of fig 1 shows the plate after protein staining. The pattern shows over 50 distinct precipitin lines: the line corresponding to antigen 60 is

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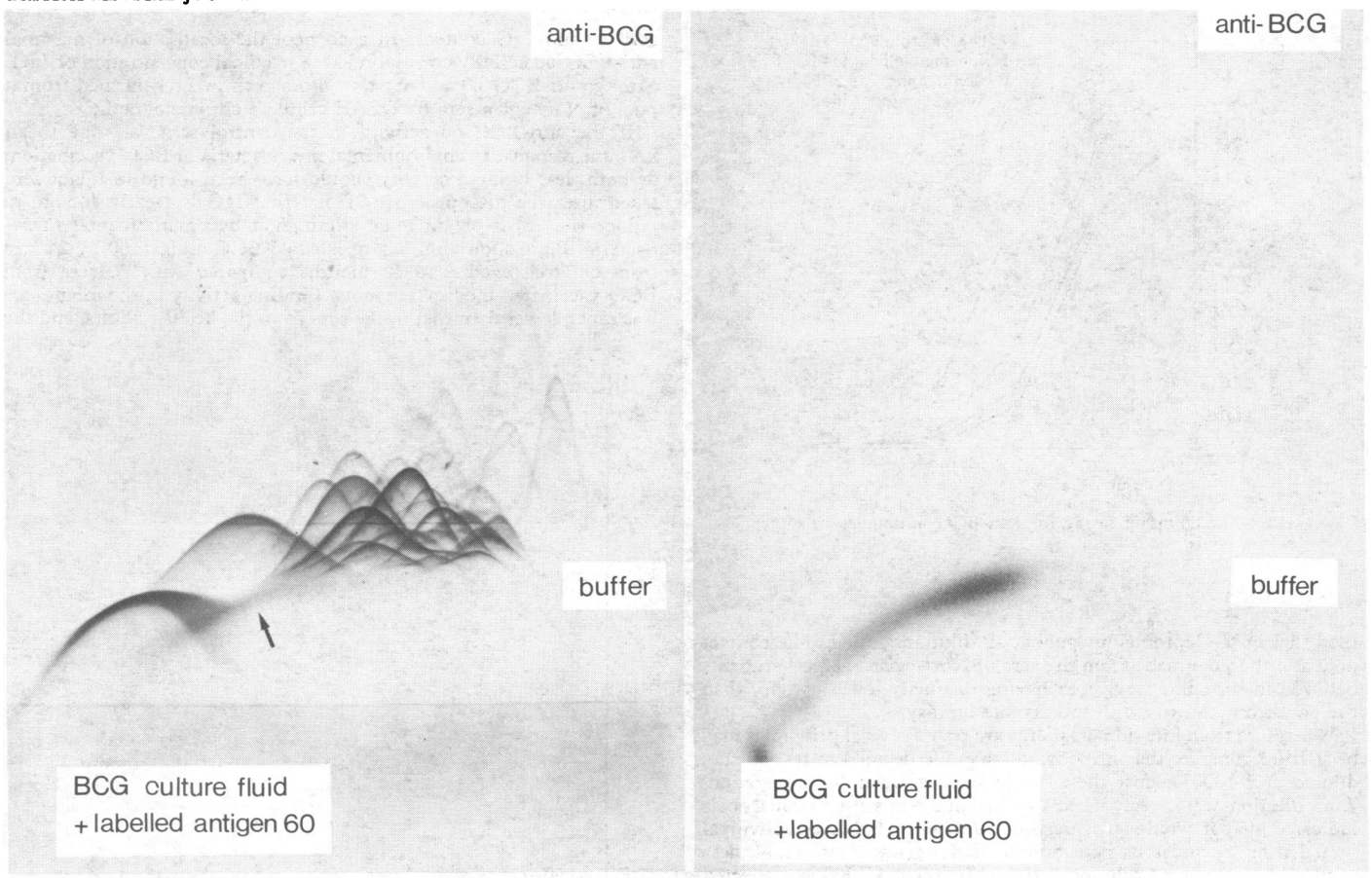

FIG 1—Crossed immunoelectrophoresis of concentrated BCG culture fluid mixed with ^{125}I-labelled BCG antigen 60 against anti-BCG. Left part shows plate after protein staining, right part after autoradiography. Radioactivity was shown in a single precipitin line corresponding to antigen 60 (arrowed).

marked with an arrow. The right part shows autoradiography of the same plate: a single line appeared on the film with the typical form and position of antigen 60. When the labelled BCG antigen 60 was gel filtered through a Sephadex G200 column the bulk of the radioactivity appeared in the void volume. By sensitive RIA procedures with specific anti-BCG-60 and polyvalent anti-BCG antibodies only trace amounts of contaminating labelled antigens could be shown behind the void volume peak.[10] Fractions corresponding to the centre of the void volume peak were used as labelled BCG antigen 60 in all tests.

RIA

Since antibodies against BCG antigen 60 occur in normal human sera (see below) and in rabbits immunised with human IgG, antibody-bound BCG antigen 60 could not be separated from free BCG antigen 60 by adding rabbit antihuman immunoglobulin. The RIA based on binding of antibody-bound antigen to protein-A-containing staphylococci[12] was therefore used as described in detail elsewhere.[10] Briefly, each tube contained 100 µl of the appropriate serum dilution and 100 µl of labelled BCG antigen 60. They were incubated for 30 minutes at 20°C before the addition of 2 ml 1% formalinised staphylococci of the Cowan I strain (NCTC 85308). All dilutions of unlabelled (cold) and labelled proteins and of antisera were made in protein containing RIA buffer. After mixing the tubes were spun at 1500 *g* for 20 minutes and the radioactivity was determined in the bacterial pellet. All values are given as mean values of double tests.

SERA

To obtain information on the overall antibody response in groups of individuals four serum pools were tested. The lepromatous serum pool contained sera from 43 patients with lepromatous or borderline lepromatous (LL, LI, and BL) leprosy.[1 13] The tuberculoid pool contained sera from 40 patients with tuberculoid (TT, TT/BT, and BT) leprosy. The contact pool contained sera from 30 Ethiopians who were known to have had contact with patients with leprosy but who had no clinical symptoms of leprosy. The fourth pool contained sera from 25 healthy BCG-vaccinated Norwegian medical students.

Information on the variation in antibody response among people in each group was obtained by testing individual samples of serum and comparing them with samples from selected controls. These sera were obtained at the Armauer Hansen Research Institute (AHRI) from 29 patients with lepromatous leprosy and 20 patients with tuberculoid leprosy attending the Addis Ababa Leprosy Hospital. The patients had been treated with dapsone for under six months. The control sera came from seven healthy staff members at the AHRI; seven Ethiopians with cutaneous leishmaniasis, including three with the diffusa form; and 36 healthy BCG-vaccinated Norwegian medical students. In addition 17 sera from patients with polyclonal hypergammaglobulinaemia (IgG content over above 25 g/l) were picked at random from sera investigated routinely in our department in Oslo for characterisation of immunoglobulin abnormalities, and eight sera were obtained at the Addis Ababa Tuberculosis Centre from patients with active pulmonary tuberculosis. The cases were recently diagnosed and verified bacteriologically and by *x*-ray examination. Twenty-four infant sera were obtained from children aged 4 to 16 months at the department of paediatrics, Ullevaal Hospital. Three sera were obtained from patients with antibody deficiency syndrome (IgG concentration $<0{\cdot}2$ g/l) before substitution treatment.

Results

Fig 2 shows the anti-BCG-60 antibody content in the four serum pools. Antibody activity is expressed as the amount of labelled BCG antigen 60 bound to staphylococci by a series of tenfold dilutions of the serum pools. The lepromatous pool had considerable binding activity with maximal uptake at dilutions of both 1/10 and 1/100. Binding activity then decreased and the titre was 10^5. The tuberculoid pool also showed considerable binding activity, but it was less pronounced

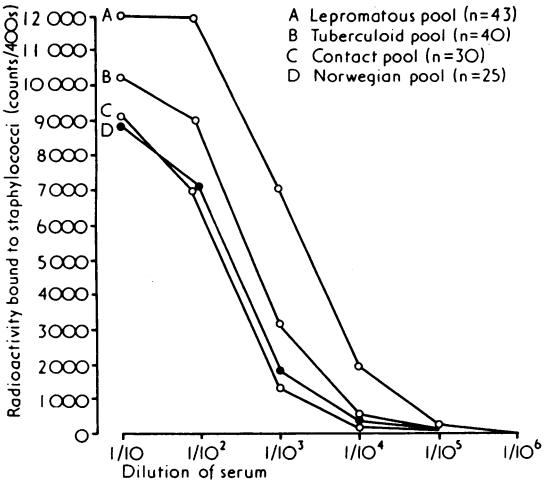

FIG 2—Radioimmunoassay for anti-BCG antibodies in four serum pools.

than that in the lepromatous pool at all dilutions tested. The contact pool and the pool made from the sera of Norwegian medical students behaved in the same way, both having distinctly lower activity than the pool from patients with tuberculoid leprosy.

When sera from individual patients are tested several principles may be used to compare the antibody activity. We decided to test all sera diluted at 1/1000 against the same labelled antigen 60 preparation. This dilution was selected because the findings with various pools indicated that it would give maximal difference between individual sera. In the lepromatous pool binding activity was considerably less at this dilution than at dilution 1/100. Dilution at 1/1000 was selected to show binding activity in individual sera, which was higher than in the pool, since such information could not be obtained in tests at dilution 1/100, where the binding activity of the lepromatous serum pool had already reached the maximal value. The results (fig 3) are expressed as relativ : antibody activity in each serum diluted at 1/1000 compared with the maximal binding activity of a monospecific anti-BCG-60 antiserum.

Fig 3 shows again that the binding activity of the lepromatous pool was stronger than that in the tuberculoid pool, which in turn was stronger than that in the contact pool. The response in 29 patients with lepromatous leprosy showed a striking variation from very strong to negligible activity, the median value corresponding closely to the activity of the lepromatous pool. The median value of 20 sera from patients with tuberculoid leprosy was lower. Again, the variation between the antibody content of individual sera was striking. In five of the 20 tuberculoid leprosy sera the anti-BCG 60 activity was above the median for the lepromatous group.

The four control groups (sera from healthy staff members at AHRI, patients with cutaneous leishmaniasis, Norwegian medical students, and Norwegians with polyclonal increased concentration of IgG) showed, with the exception of a single serum, a very similar pattern, and their median values corresponded closely to the activity of the contact pool.

Although there were only eight samples from patients with active pulmonary tuberculosis, there was again a striking variation in the anti-BCG-60 content of individual sera, and the median value in this group was even higher than that in the lepromatous leprosy group. We then sought an explanation for the aberrant behaviour of the single serum marked with an arrow in fig 3. The serum was picked at random as a control because of polyclonal increased concentration of IgG, but further examination of the clinical records showed that the patient had recently suffered from pulmonary tuberculosis.

There was considerable anti-BCG-60 activity in the contact pool made from Ethiopian sera and similar activity in the pool of sera from healthy BCG-vaccinated Norwegian medical students (fig 2). The staphylococci RIA was originally considered to detect binding of antigen by only IgG antibodies.[12] Later it was shown that some monoclonal IgM and IgA proteins were bound to protein-A-containing staphylococci,[14] and the assay is therefore also expected to detect antibodies of the IgM2 and IgA2 subclasses.[15] Samples from the lepromatous pool, the tuberculoid pool, and the contact pool were subjected to rate zonal ultracentrifugation on a sucrose density gradient,[14] and the fractions obtained were tested for anti-BCG-60 activity and IgG content. In each pool the localisation of maximal anti-BCG-60 activity corresponded to maximal concentration of IgG. Strong anti-BCG-60 activity was also shown in IgG purified from a pool of Norwegian sera by DEAE cellulose chromatography.

If the anti-BCG-60 activity in the control sera was due to an immune response to environmental mycobacteria or BCG vaccination, or both, less binding activity should have been found in infant sera taken after the disappearance of maternal IgG but early enough to reduce the effect of antigenic stimulation by environmental mycobacteria. Fig 4 shows binding of labelled BCG antigen 60 by 24 sera from children aged 4 to 16 months compared with 20 sera from BCG-vaccinated medical students. Binding activity in the infant sera was much lower than that in the sera from the healthy adults, and the

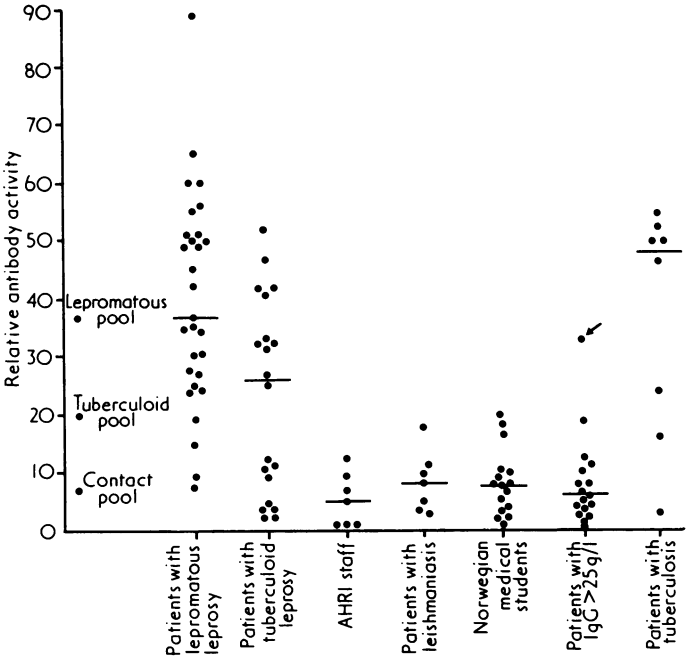

FIG 3—Radioimmunoassay for anti-BCG antibodies in individual sera from patients with lepromatous and tuberculoid leprosy, four control groups, and patients with tuberculosis. All sera were tested in dilution 1/1000. Relative antibody activity indicates binding activity as percentage of maximal binding activity by a monospecific anti-BCG-60 anti-serum. Horizontal bars indicate median values.

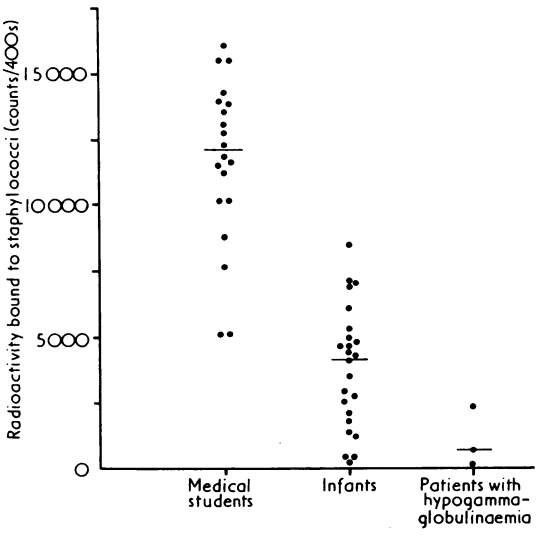

FIG 4—Anti-BCG-60 activity in sera from healthy, BCG-vaccinated Norwegian medical students compared with sera from infants aged 4 to 16 months and three patients with pronounced hypogammaglobulinaemia.

activity in three patients with humoral immunodeficiency syndrome was even lower.

Discussion

Current textbooks and reviews usually state that antibody formation against mycobacterial antigens is considerable in lepromatous leprosy and absent or very low in tuberculoid leprosy. This description seems to be part of the current view of leprosy as a spectral disease. In their study on the antibody content in sera from patients with leprosy based on double diffusion tests against an antigen prepared from *M duvalii*, Myrvang *et al* found that one of the 50 sera from the tuberculoid group (TT, TT/BT, and BT) gave a positive reaction.[16] There was a gradual increase in the frequency of precipitin-positive sera and in the number of demonstrable precipitins towards the lepromatous end of the spectrum, and 39 of the 53 LL sera (73·6%) were positive in the test.

Axelsen *et al* used CIE with intermediate gel to investigate the antibody content of leprosy sera in comparison with a BCG/anti-BCG reference system with 19 defined components.[17] Antigen 17 in their paper corresponds to our BCG antigen 60. Twelve out of 16 lepromatous sera and two out of 24 sera from the tuberculoid group showed activity against this antigen. In their investigation plates with patient serum in the intermediate gel were compared with control plates in which the intermediate gel contained serum from a healthy Dane who had never had leprosy or tuberculosis but who had been vaccinated with BCG as a child and gave a positive reaction on tuberculin testing. Antibodies were thus shown in patients' sera only when they were significantly stronger than in the selected control serum.

By using more sensitive techniques additional information may be obtained, since the remaining sera with low activity can also be evaluated and directly compared with each other. Fig 3 showed that there was a considerable variation in anti-BCG-60 activity among individual sera both in the lepromatous and the tuberculoid group. The antibody activity in the lepromatous group was distinctly higher, but in five of the 20 tuberculoid sera the anti-BCG-60 activity was above the median for the lepromatous group.

Our study shows that sera from patients with tuberculoid leprosy are also highly interesting with regard to antibody content. Furthermore, it is a new observation that anti-BCG-60 activity in tuberculoid leprosy is distinctly higher than in the various control groups. This was shown by the higher activity in the tuberculoid serum pool than in the two control pools (see fig 2) and by comparison between individual sera in the tuberculoid leprosy group and control groups.

The findings in patients with pulmonary tuberculosis were strikingly similar to those in patients with leprosy. Two points are particularly relevant: the large variation in anti-BCG-60 content in individual sera and the median value of the tuberculous group were even higher than in lepromatous leprosy. Various antigenic components of a protein nature of *M tuberculosis* can probably be labelled with ^{125}I. This may provide a basis for sensitive RIA procedures with greater specificity than conventional passive haemagglutination assays for antibodies against mycobacterial polysaccharides.

M leprae is an obligate intracellular parasite. Cell-mediated immune reactions are therefore expected to be of main importance in protective immunity.[18] The antibodies occurring in serum have attracted less interest recently apart from being acknowledged as the cause of erythema nodosum leprosum (ENL), which is recognised as a typical immune complex disease.[19 20] Our observations indicate that this view may need to be revised. The antibody content of leprosy sera should be tested with sensitive RIA techniques, and explanations for the variation in antibody content should be sought by carefully correlating clinical features with the antibody content against defined mycobacterial antigens.

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References

1 Ridley, D S, and Jopling, W H, *International Journal of Leprosy*, 1966, **34**, 255.
2 Turk, J L, and Bryceson, A D M, *Advances in Immunology*, 1971, **13**, 209.
3 Turk, J L, and Waters, M F R, *Clinical and Experimental Immunology*, 1971, **8**, 363.
4 Myrvang, B, *et al*, *Clinical and Experimental Immunology*, 1973, **14**, 541.
5 Myrvang, B, *Journal of Oslo City Hospitals*, 1975, **25**, 3.
6 Closs, O, *et al*, *Scandinavian Journal of Immunology*, 1975, **4**, suppl No 2, p 173.
7 Kronvall, G, *et al*, *International Journal of Leprosy*, 1975, **43**, 299.
8 Harboe, M, *et al*, *Scandinavian Journal of Immunology*, 1976, **5**, 861.
9 Janicki, B W, *et al*, *Infection and Immunity*, 1976, **13**, 425.
10 Harboe, M, *et al*, *Infection and Immunity*, 1977, **16**, 662.
11 Axelsen, N H, Krøll, J, and Weeke, B, *Scandinavian Journal of Immunology*, 1973, **2**, suppl No 1.
12 Jonsson, S, and Kronvall, G, *European Journal of Immunology*, 1974, **4**, 29.
13 Ridley, D S, and Waters, M F R, *Leprosy Review*, 1969, **40**, 143.
14 Harboe, M, and Følling, I, *Scandinavian Journal of Immunology*, 1974, **3**, 471.
15 Saltvedt, E, and Harboe, M, *Scandinavian Journal of Immunology*, 1976, **5**, 1103.
16 Myrvang, B, *et al*, *Acta Pathologica et Microbiologica Scandinavica*, Sect B, 1974, **82**, 701.
17 Axelsen, N H, *et al*, *Infection and Immunity*, 1974, **9**, 952.
18 World Health Organisation, Technical Report Series, No 519. Geneva, WHO, 1973.
19 Wemambu, S N C, *et al*, *Lancet*, 1969, **2**, 933.
20 Sudarsanam, A, *et al*, *African Journal of Medical Sciences*, 1971, **2**, 319.

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