INTERNATIONAL JOURNAL OF LEPROSY Volume 53, Number 2
Printed in the U.S.A.

Changes in Circulating Antibody Levels to the Major Phenolic Glycolipid During Erythema Nodosum Leprosum in Leprosy Patients[1]

Alberto Andreoli, Sara J. Brett, Philip Draper, Sheila N. Payne, and Graham A. W. Rook[2]

Erythema nodosum leprosum (ENL) is an acute reactional episode which occurs only in patients at or close to the lepromatous end of the leprosy spectrum. Although it can develop in untreated patients, ENL most commonly presents in patients following effective chemotherapy ([22]). Episodes of ENL are characteristically associated with widespread crops of small erythematous and indurated subcutaneous nodules, mainly on the face and limbs, clinically accompanied by malaise and fever. In addition, these ENL reactions frequently occur at one or several other sites, resulting in neuritis, arthritis, orchitis, uveitis, lymphadenopathy, edema of extremities, or albuminuria ([16]). Histologically ENL is predominantly a polymorphonuclear leukocyte inflammatory reaction within pre-existing lepromatous lesions and associated with vasculitis and disintegration of many of the large foamy macrophages ([13]).

ENL has been regarded as having an immune complex etiology, and it has been claimed that levels of circulating immune complexes are raised during ENL episodes ([14]). However, a more comprehensive study ([1]) failed to confirm that these levels in ENL patients were higher than in other lepromatous patients, although they were higher in lepromatous cases with or without ENL than in tuberculoid patients. On the other hand, the same authors ([1]) did demonstrate a significant correlation between ENL episodes and raised levels of the C3 breakdown product C3d. This was thought to be compatible with an extravascular occurrence of complement-fixing complexes. Histological and immunohistological evidence supports this suggestion. There is deposition of immunoglobulin and complement ([19, 24]) and an intense perivascular infiltration with polymorphs ([19, 23, 24]).

In the present study, antibody levels to phenolic glycolipid I (PGI) from *Mycobacterium leprae* and also to antigens in a soluble cell-free extract of *M. leprae* were monitored before, during, and following ENL reactions. Antibodies to PGI are found in sera from leprosy patients but not in sera from normal individuals or from patients infected with other mycobacteria ([3, 7, 27]). Such antibodies are directed predominantly toward the specific trisaccharide portion of the glycolipid ([5, 9, 29]). The antibodies to the glycolipid produced in humans appear to be predominantly of the IgM class ([28]).

MATERIALS AND METHODS

Patients. Twelve patients with lepromatous leprosy and undergoing ENL were included in this study as part of a larger longitudinal study by one of us (AA) on the clinical, histological, and immunological features of patients with multibacillary leprosy at Victoria Hospital, Dichpalli, Andhra Pradesh, India. All of the patients were clinically and histologically graded on the basis of the Ridley-Jopling classification ([17, 18]). Skin slit-smears from six sites were examined and the average calculated for determining the bacterial and morphological indices (BI and MI) ([22]). The Table sum-

[1] Received for publication on 6 June 1984; accepted for publication in revised form on 29 January 1985.

[2] A. Andreoli, M.D., and G. A. W. Rook, M.D., Department of Pathology, Middlesex Hospital Medical School, 67-73 Riding House Street, London W1P, 7D; S. J. Brett, Ph.D.; S. N. Payne, H.N.C.; and P. Draper, D.Phil., National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.
Reprint requests to Dr. Brett.

marizes some of the relevant clinical and bacteriological information on each patient. ENL was diagnosed clinically and confirmed histologically ([13]). The Table shows that ENL was usually characterized by skin lesions and fever, often accompanied by arthritis, neuritis, and swollen lymph nodes. Most of the patients had received little or only intermittent treatment prior to admission to Victoria Hospital. As shown in The Table all patients were treated with dapsone (100 mg/day) following diagnosis; the majority of patients also received clofazimine (100 mg/day) and patient 9 also received rifampin (600 mg/day for two weeks). The majority of the patients were admitted to Victoria Hospital with ENL and were treated with prednisone and/or thalidomide as required (The Table). Patient 7 developed ENL while in the hospital.

Antigens. The PGI was purified from supernatants generated during the preparation of purified suspensions of *M. leprae* from the livers and spleens of experimentally infected nine-banded armadillos ([25]). The initial extraction of lipids procedure has recently been described in detail ([8]), and the glycolipid was further purified by column and thin-layer chromatography ([15]). The material obtained was without detectable contaminants, as judged by thin-layer chromatography, and had identical physical and chemical properties to the material described by Hunter and Brennan ([11]).

A soluble antigenic extract from purified *M. leprae* suspensions was prepared by ultrasonication as previously described ([20]). The protein concentration was measured by the method of Lowry, *et al.* ([12]).

Serum samples. Serum was collected from patients at Victoria Hospital, during and following ENL episodes, by Dr. A. Andreoli. The samples were stored at −20°C, transported to the United Kingdom on dry ice, and then stored until use at −70°C. All sera sequentially collected from the same patients were tested on the same occasion in the U.K.

Enzyme-linked immunosorbent assay. The PGI was solubilized in sodium deoxycholate (1 mg/ml in 0.01 M phosphate buffered saline), and added to polyvinyl chloride microtiter plates (Dynatech Laboratories Inc., Billingshurst, Sussex, U.K.) at a concentration of 4 μg/ml. The plates were incubated for 18 hr at 37°C. Soluble *M. leprae* antigen was diluted in bicarbonate-carbonate (0.05 M) buffer (pH 9.6) at a protein concentration of 2 μg/ml and added to polystyrene microtiter plates (Dynatech) for 2 hr at room temperature. The rest of the assay was performed as described previously by Brett, *et al.* ([3]). The conjugates used were goat anti-human IgM, IgG, and IgA (μ-, γ-, and α-chain specific) which were peroxidase linked and used at optimal concentrations of 1:1000 (DAKO PATTS, High Wycombe, Bucks, U.K. raised without the use of Freund's complete adjuvant). All sera were tested in triplicate and at several dilutions. The results are presented using a serum concentration of 1:20, although a similar pattern was found at 1:10 and 1:40 dilutions. The absorbance value obtained with a pooled batch of normal human serum from the U.K. was subtracted from the absorbance obtained with the patients' sera. The absorbance value for the batch of normal human serum used was 0.12 ± 0.09 (mean ± S.D.).

Competitive inhibition experiments were performed with samples from each patient studied to check the specificity of the binding to the glycolipid. In all cases antibody binding to the PGI was significantly inhibited by preincubating the sera with the PGI or with the free terminal disaccharide, using the method described previously ([5]).

The antibody levels to the PGI and to the soluble *M. leprae* extract were compared prior to and during ENL episodes for each patient. Where possible, the mean antibody level was calculated from several samples taken when the patient was not undergoing ENL. However, only time points within a few weeks of a given ENL episode were used in order to reduce the effect of possible long-term changes in antibody levels, such as a fall in antibody levels associated with long-term chemotherapy. The antibody level during ENL was calculated as the mean absorbance of the serum samples taken during a single episode of ENL.

RESULTS

The IgM anti-PGI antibody levels and the IgM, IgG, and IgA antibody levels to soluble *M. leprae* antigen were studied in leprosy patients undergoing ENL reactions. Figure 1 shows the results obtained using the PGI

as antigen and sera from two representative patients from the group of 12 studied. Sequential serum samples were taken from the patients and the anti-PGI antibody level plotted against the time the sample was taken from the day of admission to the hospital onward. Figure 1A shows the results from patient 1 who was admitted with ENL and had a low IgM antibody level to the glycolipid. As the patient recovered from ENL the anti-PGI level increased quite rapidly. While the patient did not have ENL, the anti-PGI antibody levels did not show dramatic day-to-day fluctuations (from day 6–day 25). Subsequent ENL episodes in this patient were also accompanied by low anti-PGI antibody responses, even when the patient was receiving treatment with prednisone or thalidomide.

A similar pattern was observed with patient 8. A marked decrease in anti-PGI antibody levels occurred at the time of each ENL episode. When the patient had recovered from the ENL reaction, however, the antibody levels usually increased (Fig. 1B).

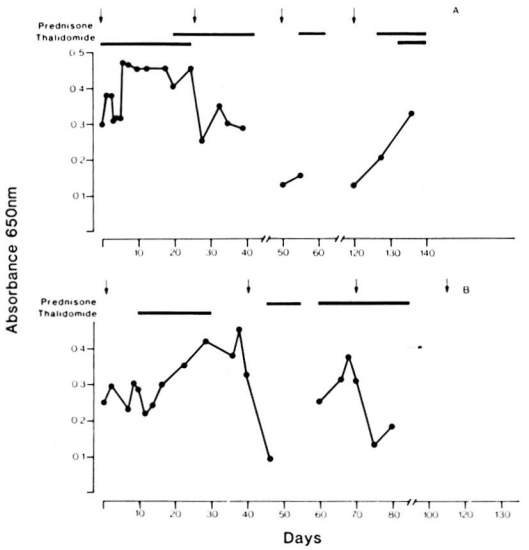

FIG. 1. IgM antibody levels to the phenolic glycolipid I antigen in Patient 1 (A) and in Patient 8 (B), where several ENL episodes occurred over a period of 4–5 months. Arrow indicates day of onset of each ENL episode. Serum dilution 1:20.

Figure 2 shows the changes in antibody level to PGI for each ENL episode for each patient studied. The mean antibody level to PGI when the patients did not have ENL is shown by point A. Point B gives the mean antibody level to PGI during an ENL episode, and point C represents the mean antibody level during ENL when the patient was being treated with prednisone or thalidomide. The results show that there was a consistent decrease in the IgM anti-PGI antibody response during periods of ENL, whether or not the patients were on anti-inflammatory therapy. There was, however, considerable variation in the observed decreases between individual episodes. There could be several reasons for this, including the severity of the ENL episode and possible modulation by steroids or thalidomide. When not undergoing ENL the same patients did not show large changes in antibody level over the short time periods studied. The range is shown in Figure 2 (points A) which shows the range of values obtained while the patients were not undergoing ENL.

The results may also be expressed as the percentage change of IgM levels during ENL. Using this method, there was a mean decrease in the antibody response during ENL of 60% (range 21–96%) and 54% (range 25–79%) during ENL while treated with prednisone or thalidomide.

The antibody levels (IgG, IgM, and IgA) to a soluble *M. leprae* antigenic preparation were also examined in this group of patients. No significant changes in antibody levels to the antigen occurred during ENL episodes, with or without treatment, in any of the immunoglobulin classes studied.

DISCUSSION

The results of this study indicate that the phenolic glycolipid from *M. leprae* may play an important role in the pathogenesis of ENL. A decrease in levels of antibody to the pure glycolipid, but not to a crude, soluble *M. leprae* antigen preparation, was a consistent finding in patients during the period of ENL reaction. These results are supported by recent observations by Cho, *et al.* (6) that antibody levels to PGI in leprosy patients with ENL were significantly lower than in active lepromatous leprosy patients with no ENL. This study did not however show antibody levels in the same patient with or without ENL. The reason for the observed decrease is not understood, but it could be due to the formation and deposition in the

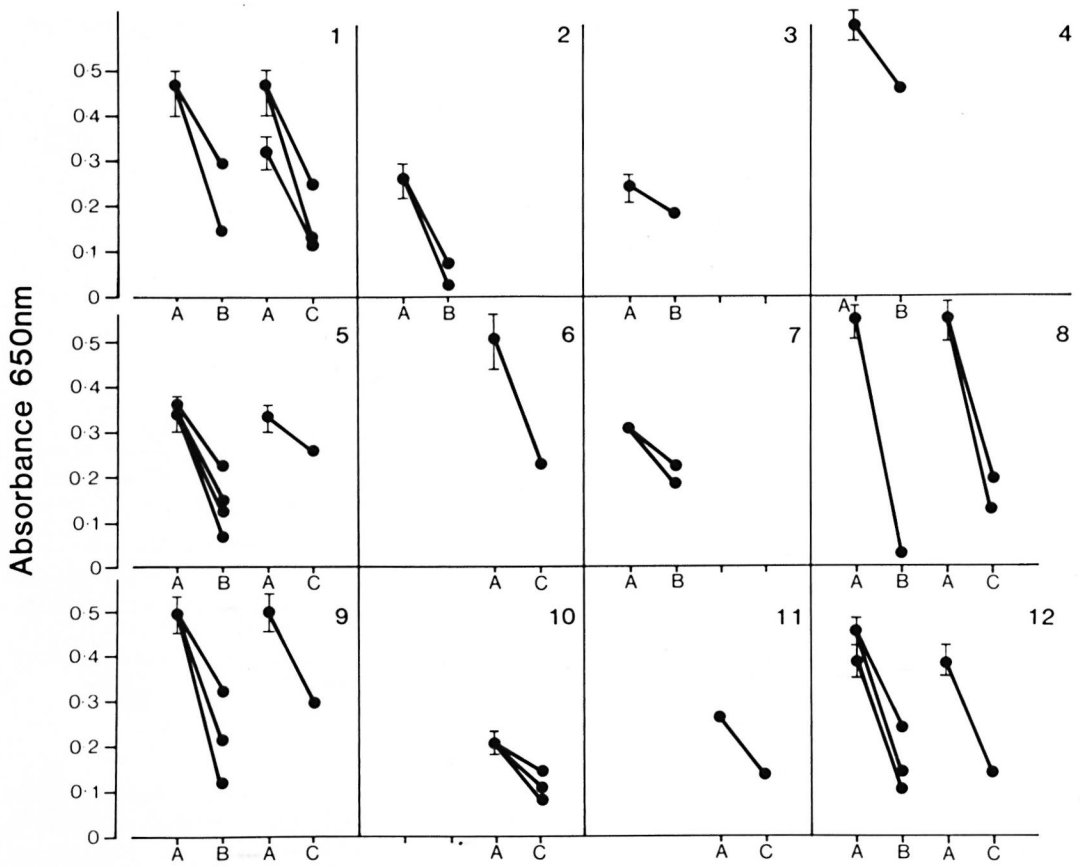

FIG. 2. IgM antibody levels to the phenolic glycolipid I antigen for 12 lepromatous leprosy patients when (A) they did not have ENL (mean ± S.D.), (B) during periods of ENL, and (C) during periods of ENL while on prednisone or thalidomide. Serum dilution 1:20.

tissues of immune complexes, composed of anti-glycolipid antibodies and the glycolipid.

Previous studies have shown that lepromatous leprosy patients have high levels of anti-PGI antibodies in their sera, even after 1–2 years of chemotherapy ([3, 7]). Large amounts of PGI have been found in infected human skin biopsies ([26]) as well as in armadillo tissue ([11]). Most of the patients in this study had a high bacterial load as shown in The Table. It has been calculated that approximately equal amounts of extracellular phthiocerol-containing lipid and bacteria (dry weight) may be found in tissues ([2]). Much of this material does not appear to be attached to the bacteria since it is found abundantly in homogenized bacteria-free supernatants of infected tissue. There is preliminary evidence, using immunoperoxidase staining of frozen sections with specific rabbit anti-glycolipid serum, that the glycolipid is found in the macrophages of infected tissue (S. Gupta, personal communication). The glycolipid may therefore be an important component of the "foam cells," characteristic in the histopathology of lepromatous leprosy, as has been previously suggested by other workers ([10]).

Interestingly, studies on the histopathology of ENL have observed that at the center of the lesions, there is often disintegration of foamy macrophages with the release of degraded bacterial material ([19]). It is suggested that following disintegration of the macrophages large amounts of glycolipid would become available for combination with circulating anti-glycolipid antibodies. This would result in a decrease in anti-glycolipid antibodies in the serum. The gly-

THE TABLE. *Clinical and bacteriological information on the lepromatous leprosy patients.*

Patient no. (Age/sex)	Classification	BI/MI	Treatment		No. ENL episodes/period of study	Type of ENL[b]	ENL treatment[a]
			Duration	Drug[a]			
1. (36 M)	BL	3.5/0.0	2 yr	D, C	5/5 months	A, F	P, T
2. (30 M)	LL	4.5/1.5	None	D	2/4 months	F, SN, A	P
3. (26 M)	BL	ND[c]	1 yr	D, C	1/2 months	F, SN	T
4. (31 F)	BL	1.3/0.0	12 yr	D, C	1/1 month	F, SN, N	P
5. (35 M)	BL	3.2/0.0	None	None	5/5 months	F, SN, N	None
6. (26 M)	BL	4.2/0.0	1 yr	D, C	1/1 month	F, SN, N	T, P
7. (45 M)	BL	4.2/0.0	None	D, C	2/5 months	F, SN	T
8. (17 M)	LL	3.8/0.0	1 yr	D, C	3/4 months	SN, N, L	P, T
9. (31 F)	LL	4.3/1.2	None	D, C, R	4/5 months	F, SN, A	P
10. (35 M)	LL	4.3/1.3	None	D, C	3/1 month	F, SN, L	T
11. (61 M)	LL	5.0/0.6	1 month	D, C	1/3 months	F, SN, A	T
12. (37 M)	BL	3.8/0.0	Intermittent for 3 yr	D, C	4/3 months	F, SN	P, T

[a] D = dapsone, C = clofazimine, R = rifampin, T = thalidomide, P = prednisone.
[b] ENL symptoms: A = arthritis, F = fever, SN = skin nodules, L = lymphadenopathy, N = neuritis.
[c] ND = Not done.

colipid is an uncharged lipophilic molecule probably resistant to lysosomal degradation, which may explain the persistence and buildup of this molecule in the tissues. In contrast the soluble *M. leprae* components, such as proteins, probably leach out of the bacteria and are broken down relatively easily by the macrophages. Thus such antigens would not persist and build up in the macrophages. This may be one reason why during ENL reactions the antibody levels to soluble *M. leprae* antigens do not change, while those to the glycolipid decrease.

Another feature of the histopathology of ENL reactions is that following degeneration of foamy macrophages and deposition of immune complexes composed of IgM, IgG, and complement components of the classical pathway, there is usually marked infiltration of the lesions with polymorphonuclear leukocytes (neutrophils) ([19, 23]). We have observed that subcutaneous injection of glycolipid into mice elicits a strong nonspecific inflammatory response at 24–48 hr composed predominantly of polymorphs ([4]). Thus, the polymorph infiltration found in ENL reactions may be at least partially in response to the glycolipid release from degenerating macrophages.

Other workers have examined circulating levels of mycobacterial antibodies during ENL reactions. Antibody levels to soluble antigens of several mycobacterial species did not change appreciably in the sera of patients undergoing ENL reactions and receiving thalidomide treatment ([21]). This agrees with the results obtained in the present study with soluble antigen, which is likely to contain little of the water-insoluble phenolic glycolipid.

We have not calibrated the ELISA assay in terms of the quantity of immunoglobulin binding to a given weight of the glycolipid, so we are not at present in a position to prove that enough antibody can be absorbed by the quantities of glycolipid present in typical ENL lesions to account for the striking falls in circulating levels. However, as discussed above, the quantities of glycolipid in the tissues are so large that the suggestion seems reasonable, and seems the only simple explanation of our findings.

SUMMARY

Circulating antibody levels to the phenolic glycolipid from *Mycobacterium leprae* and soluble *M. leprae* antigens were monitored before, during and following ENL episodes in 12 patients. It was observed that during ENL reaction, there was a fall in circulating antibody levels to the phenolic glycolipid but not to the soluble antigens from *M. leprae.* When the patients had recovered from their ENL reactions, the anti-glycolipid antibody levels usually increased again

to levels similar to those observed before the ENL reaction.

RESUMEN

Se cuantificaron los niveles de anticuerpos circulantes contra el glicolípido fenólico y contra antígenos solubles del *Mycobacterium leprae,* antes, durante, y después de los episodios reaccionales tipo ENL en 12 pacientes. Se observó que durante la reacción ENL hubo una caída en los niveles de anticuerpos circulantes contra el glicolípido fenólico pero no contra los antígenos solubles del *M. leprae.* Cuando los pacientes se hubieron recuperado de sus reacciones ENL, los niveles de anticuerpos contra el glicolípido generalmente aumentaron otra vez hasta alcanzar valores similares a los observados antes de la reacción ENL.

RÉSUMÉ

Les taux d'anticorps circulants contre le glycolipide phénolique extrait de *Mycobacterium leprae* et contre les antigènes solubles de *M. leprae* ont été suivis avant, pendant, et après des épisodes d'ENL survenus chez 12 malades. On a observé qu'au cours de la réaction d'ENL, il se produisait une chute dans les taux d'anticorps circulants contre le glycolipide phénolique, mais non aux anticorps dirigés contre les antigènes solubles de *M. leprae.* Lorsque les malades ont surmonté leur réaction d'ENL, les taux d'anticorps antiglycolipides retournent généralement au niveau observé avant la réaction d'ENL.

Acknowledgments. We would like to thank Dr. M. J. Colston and Dr. R. J. W. Rees for helpful discussion and advice during this study. One of us (AA) gratefully acknowledges the receipt of a grant from the British Leprosy Relief Association (LEPRA) while working at Victoria Hospital, Dichpalli, India, and the Middlesex Hospital, London.

REFERENCES

1. Bjorvatn, B., Barnetson, R. St. C., Kronvall, G., Zubler, R. H. and Lambert, P. H. Immune complexes and complement hypercatabolism in patients with leprosy. Clin. Exp. Immunol. **26** (1976) 388–396.
2. Brennan, P. J. The phthiocerol-containing surface lipids of *M. leprae*—a perspective of past and present work. Int. J. Lepr. **51** (1984) 387–396.
3. Brett, S. J., Draper, P., Payne, S. N. and Rees, R. J. W. Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. Clin. Exp. Immunol. **52** (1983) 271–279.
4. Brett, S. J., Lowe, C., Payne, S. N. and Draper, P. Phenolic glycolipid I of *Mycobacterium leprae* causes non-specific inflammation but has no effect on cell-mediated responses in mice. Infect. Immun. **46** (1984) 802–808.
5. Brett, S. J., Payne, S. N., Draper, P. and Gigg, R. H. Analysis of the major antigenic determinants of the characteristic phenolic glycolipid from *Mycobacterium leprae.* Clin. Exp. Immunol. **56** (1984) 89–96.
6. Cho, J. N., Fukiwara, T., Hunter, S. W., Rea, T. H., Gelber, R. H. and Brennan, P. J. Use of an artificial antigen containing the 3,6-di-*O*-methyl β-D-glucopranosyl epitope for the serodiagnosis of leprosy. J. Infect. Dis. **150** (1984) 311–322.
7. Cho, S. N., Yanagihara, D. L., Hunter, S. W., Gelber, R. H. and Brennan, P. J. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. Infect. Immun. **41** (1983) 1077–1083.
8. Draper, P., Payne, S. N., Dobson, G. and Minnikin, D. E. Isolation of a characteristic phthiocerol dimycocerosate from *M. leprae.* J. Gen. Microbiol. **129** (1983) 859–863.
9. Fujiwara, T., Hunter, S. W., Cho, S.-N., Aspinall, G. O. and Brennan, P. J. Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. Infect. Immun. **43** (1984) 245–252.
10. Fukunishi, Y., Kearney, G. P., Whiting, J., Jr., Manders, W. W., Walsh, G. P., Binford, C. H., Meyers, W. M. and Johnson, F. B. Biochemical investigations of the peribacillary substance of *Mycobacterium leprae.* Int. J. Lepr. **51** (1983) 668–669.
11. Hunter, S. W. and Brennan, P. J. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147** (1981) 728–735.
12. Lowry, D. H., Rosebrough, N. J., Farr, A. L. and Randall, E. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193** (1951) 265–275.
13. Mabalay, M. C., Helwig, E. B., Tolentino, J. G. and Binford, C. H. The histopathology and histochemistry of erythema nodosum leprosum. Int. J. Lepr. **33** (1965) 28–49.
14. Moran, C. J., Turk, J. L., Ryder, G. and Waters, M. F. R. Evidence for circulating immune complexes in lepromatous leprosy. Lancet **2** (1972) 572–573.
15. Payne, S. N., Draper, P. and Rees, R. J. W. Serological activity of purified glycolipid from *M. leprae.* Int. J. Lepr. **50** (1982) 220–221.
16. Ridley, D. S. Reactions in leprosy. Lepr. Rev. **40** (1969) 77–81.
17. Ridley, D. S. and Jopling, W. H. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. **34** (1966) 255–273.
18. Ridley, D. S. and Waters, M. F. R. Significance of variations within the lepromatous group. Lepr. Rev. **40** (1969) 143–152.
19. Ridley, M. J. and Ridley, D. S. The immunopathology of erythema nodosum leprosum: The

role of extravascular complexes. Lepr. Rev. **54** (1983) 95–107.
20. SMELT, A. H., REES, R. J. W. and LIEW, F. Y. Induction of delayed-type hypersensitivity to *M. leprae* in healthy individuals. Clin. Exp. Immunol. **44** (1981) 501–506.
21. ULRICH, M., PINARDI, M. E. and CONVIT, J. A study of antibody response in leprosy. Int. J. Lepr. **37** (1969) 22–27.
22. WATERS, M. F. R., REES, R. J. W. and SUTHERLAND, I. Chemotherapeutic trials in leprosy. 5. A study of methods used in clinical trials in lepromatous leprosy. Int. J. Lepr. **35** (1967) 311–335.
23. WATERS, M. F. R., TURK, J. L. and WEMAMBU, S. N. C. Mechanisms of reactions in leprosy. Int. J. Lepr. **39** (1971) 417–428.
24. WEMAMBU, S. N. C., TURK, J. L., WATERS, M. F. R. and REES, R. J. W. Erythema nodosum leprosum: A clinical manifestation of the Arthus phenomenon. Lancet **2** (1969) 933–935.
25. WORLD HEALTH ORGANIZATION. Purification of *M. leprae.* Report of the fifth meeting of the Scientific Working Group on the immunology of leprosy (IMMLEP), Geneva 24–26 June 1980. TDR/IMMLEP—SWG (5)/80.3.
26. YOUNG, D. B. Detection of mycobacterial lipids in skin biopsies from leprosy patients. Int. J. Lepr. **49** (1981) 198–204.
27. YOUNG, D. B. and BUCHANAN, T. M. A serological test for leprosy with a glycolipid specific for *M. leprae.* Science **221** (1983) 1057–1059.
28. YOUNG, D. B., DISSANAYAKE, S., MILLER, R. A., KHANOLKAR, S. R. and BUCHANAN, T. M. Humans respond predominantly with IgM immunoglobulin to the species specific glycolipid of *M. leprae.* J. Infect. Dis. **149** (1984) 870–873.
29. YOUNG, D. B., KHANOLKAR, S. R., BARG, L. L. and BUCHANAN, T. M. Generation and characterization of monoclonal antibodies to the phenolic glycolipid of *Mycobacterium leprae.* Infect. Immun. **43** (1984) 183–188.