# Comparison of Various Preparations of *Mycobacterium leprae* and Other Mycobacteria by Lymphocyte Stimulation [1]

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Lymphocytes from people sensitized to *M. leprae*, or lymphocytes with a specific lack of response to the pathogen as those of lepromatous leprosy patients ([11]) can be used to study the similarity and/or difference between *M. leprae* and other microbes.

For taxonomic classification this method has no advantage over biochemical, serologic or growth related characteristics. However, the lymphocyte stimulation test may detect relationships of great clinical relevance since cell-mediated immune responses (CMI) are of paramount importance both in protective immunity ([22]) and in the development of complications in leprosy ([17]). One major obstacle for the comparison of *M. leprae* to other mycobacteria has been the shortage of leprosy bacilli. *M. leprae* has so far not been successfully grown in artificial medium. Attempts to inoculate experimental animals with the bacillus have resulted in limited multiplication ([19]), until recently when the nine-banded armadillo (*Dasypus novemcinctus*) was found to be a suitable host ([14]). In 1975, Skinsnes and co-workers ([21]) claimed to have grown *M. leprae* on ordinary media for mycobacteria supplemented with hyaluronic acid. However, no clearcut criteria for the identity of *M. leprae* exist and the conclusion has to be based on several more vague attributes of the bacilli ([20]). In the present paper, responses to different mycobacterial preparations have been measured with the *in vitro* lymphocyte stimulation test. Peripheral blood lymphocytes from leprosy patients and healthy contacts of leprosy patients were stimulated in culture with *M. leprae* from human nodules, acid-fast bacilli from *M. leprae* infected armadillos, acid-fast bacilli from LA-3 medium inoculated with *M. leprae*, *M. bovis* (BCG) and purified protein derivative (PPD) from *M. tuberculosis*.

## MATERIALS AND METHODS

**Test subjects.** Thirty-one individuals between 18 and 38 years of age were lymphocyte donors. Six had no clinical signs of leprosy and had experienced different degrees of exposure to leprosy patients. All leprosy patients were in their first year of treatment and had no other relevant diseases. Nine patients were classified as lepromatous leprosy patients (BL, LI and LL), and 16 had tuberculoid leprosy (TT, TT/BT and BT). Four of the tuberculoid leprosy patients had some degree of acute inflammation in their lesions at the time of testing, while the rest had flat, hypopigmented lesions. Two had recently recovered from a reversal reaction.

**In vitro lymphocyte stimulation.** The lymphocyte transfer test (LTT) was performed with a micromethod described earlier, using incorporation of tritiated thymidine as a measure of lymphocyte stimulation ([7, 8]).

Lymphocyte cultures were run in triplicates and the antigens to be compared were added under identical conditions to the same microculture tray. Each antigen was added to the lymphocyte cultures at three different dilutions ($10^{-1}$, $10^{-2}$ and $10^{-3}$) which generally covered the area of optimal stimulation. Large variations in the degree of clumping make it difficult to count the exact number of AFB/ml.

**Antigens.** Two different batches of human lepromin were used as the source of *M. leprae* antigen. One (GAO-612) contained washed whole bacilli from a single subcuta-

neous-nodule. This preparation is identical to the one used in earlier studies ([4, 7]). The other batch (S-75) was a pool of washed bacilli obtained from nodules of 20 different lepromatous leprosy patients. Both were produced by homogenization and differential centrifugation from fresh biopsies, as previously described ([7]). Suspensions of whole bacilli were washed three times in 0.9% NaCl with 1% human serum albumin (Kabi, Uppsala, Sweden) to decrease clumping, counted by the spot slide method ([13]) and the concentration was adjusted to $10^9$ acid-fast bacilli (AFB) per ml and stored in small aliquots at -70°C until used.

AFB from armadillos infected intravenously three years earlier with *M. leprae* from human tissues were produced by homogenization of subcutaneous tissues and were a gift from Dr. Rees in London. One batch (A) was prepared according to the method of Draper and Rees ([10]), using collagenase, trypsin and pronase to digest the infected tissues. The second batch (B) was prepared from the same material by homogenization of fresh tissue in ice cold saline to minimize destruction of surface antigens of the bacilli. The two suspensions were made up to $10^9$ AFB/ml in Dr. Rees' laboratory but due to spontaneous clumping, no comparison count could be performed in Ethiopia. AFB from LA-3 medium inoculated with *M. leprae* from human tissues and grown by Dr. Skinsnes and collaborators in Hawaii ([21]) was kindly supplied by him (HI-75 subculture 9). The bacilli were harvested in the log phase of growth (2 weeks after inoculation) and transported at 0-5°C in LA-3 medium. When received the bacilli were washed three times in saline, counted, and the concentration was adjusted to $10^9$ AFB /ml. Plates of solid Dubos medium without hyaluronic acid were inoculated with some of the washed bacilli and incubated at 37°C. After three weeks, distinctive yellow colonies containing plemorphic AFB could be observed. Suspensions were prepared from these colonies in the same way (Dubos) and were compared to the "parent" stock (HI-75). *M. bovis,* strain BCG, was prepared from commercial human vaccine produced by Glaxo Laboratories. Preservative free PPD (purified protein derivative from *M. tuberculosis,* H37RV) was obtained from Statens Seruminstitut, Copenhagen, Denmark.

**Statistical methods.** Lymphocyte stimulation was measured by incorporation of tritiated thymidine, counted in a liquid scintillation counter and recorded as mean counts per minute (cpm) in triplicates stimulated with antigen minus mean cpm in triplicates without any antigen. Usually, antigen stimulated cultures had a higher cpm than unstimulated ones. In some leprosy patients, however, the addition of antigen resulted in a lower cpm than was found in unstimulated cultures. Results to be compared were, therefore, treated in different ways: 1) the triplicated giving the largest numerical difference from unstimulated cultures, irrespective of antigen dose, called "Maximum response"; 2) the highest positive net count for stimulation, "Maximum stimulation," all antigen doses giving negative Δ cpm are excluded, 3) the same for the inhibitory type of responses, called "Maximum inhibition," with results giving positive Δ cpm excluded.

Correlations were examined by the lesser quadrant method for linear regression, as experience with a great number of correlation plots gave no indication of a regression line systematically diverging from a straight line. Plottings and calculations were done on a Hewlett-Packard 9820A computer. The method of Hald ([12]) was used to test for significance of slopes differing from 1 (the relative potency of the antigens as lymphocyte stimulators). "Degree of explanation" (D. E.) expressing the percent of variation in "y" explained by the variation in "x" is given as a measure of the variation around the calculated line of regression.

## RESULTS

The correlation plots for preparations which can be predicted to share some antigens are shown in Figure 1, and the calculations of correlations in Table 1. In the correlation plot for the two human lepromins (GAO-612 and S-75), the spread around the calculated line of regression indicates the batch-to-batch variation plus the experimental error in the test. The correlation (r:0.89, D.E.:55 for maximal responses) and the lack of seriously diverse observations in the plot, indicate no clear-cut antigenic difference between the two. The correlation for positive responses (r:0.78, D.E.:
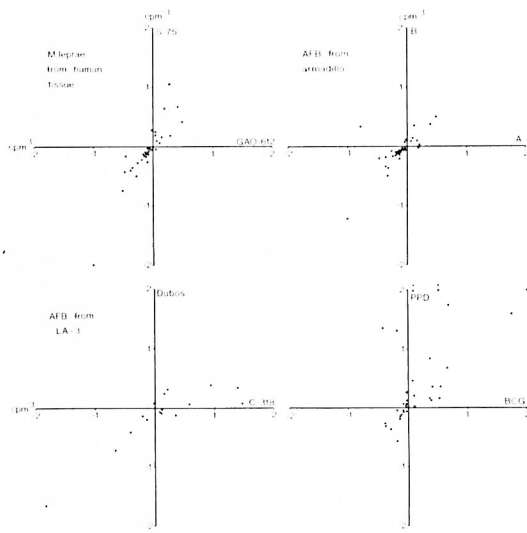
FIG. 1. Correlation plots for pairs of mycobacterial preparations which can be expected to share antigens. S-75 and GAO-612 are different batches of AFB prepared from human lepromatous nodules. Batch A are AFB from *M. leprae* infected armadillos prepared by enzyme treatment of tissues; batch B are AFB from the same source but prepared by homogenization and differential centrifugation in cold saline. HI-75 are AFB grown on LA-3 medium inoculated with *M. leprae*, and Dubos are AFB from the secondary isolate of HI-75 grown on Dubos solid medium without hyaluronic acid. BCG is *M. bovis* (Glaxo strain) and PPD is purified protein derivative from *M. tuberculosis* H37Rv. Responses are recorded as $\Delta$ cpm × $10^{-3}$.

36) is lower than for inhibitory responses (r:0.89, D.E.:55) but is probably still compatible with this conclusion.

Enzyme treatment of AFB from armadillos does not seem to change their antigenic make-up drastically. One observation, however, deviates strongly from the regression line and affects the correlation (r:0.72, D.E. :70) greatly. If this observation is left out, r will be 0.93 and D.E.:62. This deviating result was recorded in a post-reactional tuberculoid patient, and it might indicate a real antigenic difference due to the enzyme treatment, traced here by a relatively rare antigenic specificity displayed by this patient's lymphocytes.

Correlation between parent and daughter strain adopted from LA-3 grown bacilli indicates great antigenic similarity between the two (r:0.85, D.E.:43). There is, however, an interesting difference between stimulatory and inhibitory responses. For inhibitory responses, the correlation is very good (r:0.99, D.E.:87) while stimulatory responses do not correlate well (r:0.55, D.E.:14). This indicates a real antigenic difference between the two isolates grown *in vitro*.

Lymphocytes of these test subjects, which might mainly express sensitization to *M. leprae*, do not distinguish clearly between BCG and PPD (r:0.79, D.E.:38). Inhibitory responses to these antigens were too few to allow calculations of correlation.

TABLE 1. *Calculations of correlation coefficients r, and degrees of explanation D.E., in the comparison between mycobacterial preparations which can be expected to share antigens (Fig. 1).*

| Compared antigens | Maximum[a] | | Maximum stimulation[b] | | Maximum inhibition[c] | |
|---|---|---|---|---|---|---|
| | r | D.E. | r | D.E. | r | D.E. |
| *M. leprae* human GAO-612/S-75 | .89 | 55 | .78 | 36 | .89 | 55 |
| AFB armadillo A/B | .72 | 30 | .64 | 22 | .67 | 25 |
| AFB LA-3 HI-75/Dubos | .85 | 46 | .55 | 14 | .99 | 87 |
| *M. tuberculosis* BCG/PPD | .79 | 38 | .79 | 38 | .62 | 21 |

[a]"Maximum" means responses which show the largest numerical deviation from the unstimulated control whether the $\Delta$ cpm was a negative or a positive value.
[b]"Maximum stimulation" means largest positive $\Delta$ cpm.
[c]"Maximum inhibition" means the largest negative $\Delta$ cpm.

It was found that S-75, HI-75 and PPD were all significantly more potent as stimulators in LTT (p < 0.001) than GAO-612, Dubos and BCG, respectively. Enzyme treatment of AFB from armadillos did not significantly affect the relative potency of these bacilli as stimulators in LTT.

Correlation plots for the comparison of different mycobacteria are shown in Figure 2 and the results of calculations in Table 2.

Responses to BCG and human lepromin (average of responses to the two human batches) did not correlate (r:-0.35, D.E.:5). Similar degrees of difference were found between BCG on the one side and AFB from armadillo (r:-0.38, D.E.:6) and AFB from LA-3 medium (r:-0.66, D.E.:22) on the other side. Taking into consideration that the strain grown on Dubos medium inoculated with bacilli from LA-3 medium might be a contaminant from the original isolate in the LA-3 medium, individual comparisons were also made with these two strains which produced almost indentical results. Human lepromin gave significantly higher lymphocyte responses (p < 0.001) than AFB from the armadillo.

The comparison of human lepromin with AFB from armadillos (average of responses to batches A and B) revealed a large degree of antigenic similarity (r:0.94, D.E.:67). The
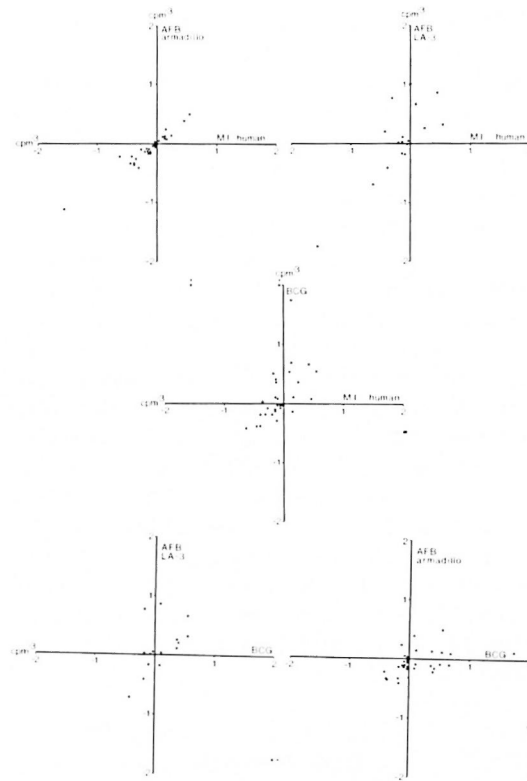


FIG. 2. Correlations plots for pairs of different mycobacterial species and for known species compared to unidentified isolates. Responses are recorded as $\Delta$ cpm × $10^{-3}$.

TABLE 2. *Calculations of correlation coefficients r, and degrees of explanation D.E., in the comparisons between different mycobacterial species and for known species compared to unidentified isolates (Fig. 2).*

| Compared antigens | Maximum[a] | | Maximum stimulation[b] | | Maximum inhibition[c] | |
|---|---|---|---|---|---|---|
| | r | D.E. | r | D.E. | r | D.E. |
| *M. leprae* human/ AFB armadillo | .94 | 67 | .83 | 43 | .93 | 64 |
| *M. leprae* human/ AFB LA-3 | .81 | 40 | .43 | 6 | .97 | 75 |
| *M. leprae* human/ BCG | –.35 | 5 | –.02 | 0 | .31 | 4 |
| BCG/ AFB armadillo | –.38 | 6 | –.03 | 0 | .24 | 2 |
| BCG/ AFB LA-3 | –.66 | 23 | –.13 | 0 | .18 | 0 |

[a]"Maximum" means responses which show the largest numerical deviation from the unstimulated control whether the $\Delta$cpm was a negative or a positive value.

[b]"Maximum stimulation" means largest positive $\Delta$ cpm.

[c]"Maximum inhibition" means the largest negative $\Delta$cpm.

effect of one single patient who responded very differently to armadillo AFB batch A and B was largely neutralized by taking the average of the two responses. The correlation is similar for the stimulatory (r:0.83, D.E.:43) and the inhibitory (r:0.93, D.E.:64) effect of the antigens.

Lymphocyte responses to AFB from LA-3 medium and their daughter strain (Dubos) correlated reasonably well with responses to human lepromin for maximal responses (r: 0.87, D.E.:50). However, calculations of correlation for inhibitory and stimulatory effects of the preparations revealed that this is largely due to an almost perfect correlation of the inhibitory effect of the bacilli, while there was virtually no correlation for the stimulatory effect (r:0.43, D.E.:6). When the calculations were done separately for the two *in vitro* grown strains against human lepromin, both correlated equally poorly with *M. leprae* with respect to stimulatory effect.

## DISCUSSION

The interaction between a whole microbe and a sample of mononuclear cells from peripheral blood is a complex one. Mycobacteria have more than 50 different molecules which are recognizable for the immune system. Kronvall *et al* ([15]) *(M. smegmatis),* Closs *et al* ([9]) *(M. lepraemurium),* and many of these molecules expose several different antigenic determinants ([16]). These antigenic determinants might stimulate different subpopulations of cells in the culture, including suppressor cells. The molecules could also act as nonspecific mitogens ([3]) or inhibitors of lymphocyte growth and stimulation ([18]). The ultimate result, the cpm recorded on termination of the culture, reflects an unknown mixture of such reactions between microbial products and the mononuclear blood cells.

Interpretation of the results for comparison of microbes must therefore be done with great caution. If responses to two antigen preparations correlate perfectly, this does not prove identity, but only means that the actual lymphocytes did not detect any difference between the two. On the other hand, microbes of the same origin can give highly different responses due to different treatment of the bacilli ([4]). A poor correlation does therefore not exclude identity but might instead reflect antigenic heterogeneity in the responses of the cell donors. This method has, however, one advantage; by using naturally sensitized individuals as blood donors, the search for relationships between microbes is limited to microbial products of some importance in the natural infections.

Two different batches of AFB from leprosy patients, one prepared from a single subcutaneous nodule and one prepared from a pool of nodules from 20 different patients, by the same procedure, behaved almost identically in the test. The deviation from a perfect correlation illustrates the technical variation in the method, which is, indeed, not negligible and also demonstrates possible antigenic variation from batch-to-batch of the same microbe.

The much lower degree of correlation between responses to whole BCG and soluble PPD is most probably not due to structural differences between PPD from *M. tuberculosis* and the corresponding molecules in *M. bovis* ([8]), but rather to additional antigens presented by whole BCG and to a different physical state of the cross-reacting antigens in the two preparations, corpuscular versus soluble.

The standard procedure for purification of AFB from infected armadillo tissues includes a step of collagenase, trypsin and pronase digestion ([10]). This treatment could damge surface proteins and thereby affect the ability of the bacilli to stimulate lymphocytes, or even change the specificity of the preparation by exposing new antigenic determinants which are hidden in the untreated preparation ([1]). Comparison between bacilli produced by the standard method and a batch from the same source produced by direct homogenization in cold saline only (as was used for the human material), came out with almost identical results in the two preparations. There was, however, one exception; a borderline tuberculoid patient who had recently had a reversal reaction, who responded to bacilli homogenized in saline with a stimulation of thymidine incorporation, but who had an inhibitory type of response to the enzyme digested preparation. For one observation in 34 a technical explanation is possible but one should also keep in mind the possibility of a real change

in a surface characteristic to which only one of our patients was specifically sensitized. On the whole, the results confirm earlier attempts to identify these bacilli as really being *M. leprae* ([14]).

Good correlation was also obtained between AFB grown by Dr. Skinsnes and coworkers on their LA-3 medium and a secondary isolate from his culture to Dubos solid medium without any hyaluronic acid added. This strongly indicates identity between the main growths in both preparations. The correlation for maximal responses between LA-3 grown bacilli and AFB from human lepromatous nodules was equally as good as the correlation between the latter and AFB from armadillos. However, looking at the results in more detail brought forward two interesting findings. While a near to perfect correlation between human lepromin and bacilli from *M. leprae* infected armadillos is disturbed by one single observation, about a third of the observations are clearly, but less dramatically, deviating from the line of regression between human lepromin and LA-3 grown bacilli. Also, in all observed deviations the cultures are stimulated by the antigens to increased thymidine incorporation. Assuming that stimulatory and inhibitory lymphocyte responses can be mediated by different antigenic specifications, we tested the two types of responses independently for correlation. For antigen-mediated inhibition of thymidine incorporation there is a perfect correlation between AFB grown on the LA-3 medium and *M. leprae* from human nodules, while the correlation for stimulatory responses, resulting in increased thymidine incorporation, is nil.

Antigen mediated inhibition of spontaneous thymidine incorporation in lymphocyte cultures had been noted by others ([2]). What exactly the phenomenon reflects is obscure, and most probably different mechanisms can give the same kind of results. It is tempting, however, to relate it to control mechanisms of the immune system, possibly suppressor cells ([5]). A strict control of the immune response seems indispensable in a disease like leprosy with a chronic stimulation of the immune system ([6]). AFB from LA-3 medium infected with *M. leprae* from humans seems to share antigens involved in such responses with *M. leprae*, which makes it an interesting candidate for

further study. It is difficult to know if the antigenic differences, illustrated by stimulation of thymidine incorporation, are too great to make *in vitro* grown AFB from LA-3 medium compatible with *in vivo* grown *M. leprae* on the other side.

The conclusions of this study are as follows:

1. AFB from armadillos infected with *M. leprae* from humans behaved almost identically to AFB prepared directly from human lepromas in the lymphocyte stimulation test, except for a somewhat lower potency of AFB from armadillos to stimulate the lymphocytes. This implies that a large standard batch could be made from armadillo material to standardize the lymphocyte stimulation technics used in different laboratories in the study of cell-mediated immunity in leprosy.

2. AFB grown on LA-3 medium inoculated with *M. leprae* from humans shares an interesting trait with bacilli from human lepromatous nodules, namely the inhibitory effect on lymphocyte reaction *in vitro*. This should be a subject for further study. With reference to the identity of the *in vitro* grown bacilli, the method has given inconclusive results.

## SUMMARY

Peripheral blood lymphocytes were stimulated *in vitro* with different mycobacterial antigen preparations and responses were measured as incorporation of tritiated thymidine. Blood donors were 9 patients with lepromatous leprosy, 16 tuberculoid leprosy patients, and 6 healthy individuals with different degrees of exposure to leprosy. The results revealed a good correlation between the responses to *M. leprae* from human sources and bacilli from armadillos inoculated with human leprosy bacilli, although the latter were less potent stimulators. Responses to BCG and PPD did not correlate with lymphocyte responses to human *M. leprae*. Acid-fast bacilli grown on LA-3 medium inoculated with human leprosy bacilli stimulated responses which did not correlate with responses to human *M. leprae* when positive responses were compared, but showed a good correlation when inhibitory effects of the bacilli on thymidine incorporation were studied. The significance

of the lymphocyte stimulation test for identification of microbes is discussed.

## RESUMEN

Se estimularon linfocitos de sangre periférica, *in vitro,* con diferentes preparaciones antigénicas micobacterianas. Las respuestas se midieron por la incorporación de timidina. 'Los donadores de sangre fueron 9 pacientes con lepra lepromatosa, 16 pacientes con lepra tuberculoide y 6 individuos sanos con diferentes grados de exposición a la lepra. Los resultados revelaron una buena correlación entre las respuestas hacia el *M. leprae* de origen humano y de armadillos inoculados con bacilos de la lepra humana, aunque los últimos fueron estimuladores menos potentes. Las respuestas al BCG y al PPD no correlacionaron con las respuestas de los linfocitos al *M. leprae* humano. Los bacilos ácido-resistentes crecidos en medio LA-3 inoculado con bacilo de la lepra humana, estimularon respuestas que no correlacionaron con las respuestas hacia el *M. leprae* humano cuando se compararon respuestas positivas, pero mostraron una buena correlacion cuando se estudiaron los efectos inhibitorios de los bacilos sobre la incorporación de timidina. Se discute el significado de la prueba de la estimulación de los linfocitos cuando se usa para la identificación de microbios.

## RÉSUMÉ

On a stimulé *in vitro* des lymphocytes du sang périphérique avec des antigènes mycobactériens divers et determiné la thymidine incorporée. Les lymphocytes ont été obtenus des donneurs suivants: 9 malades de lèpre lépromateuse, 16 malades de lèpre tuberculoïde, et 6 individus sains exposés à la lépre à des degrés différents. *M. leprae* provenant des malades et des tatous ont provoqués des activités semblables, mais l'activité stimulatrice des bacilles de l'origine humaine était inférieure. Il n'y avait pas de corrélation entre les réponses au BCG et au PPD, et la réponse à *M. leprae*. BAR cultivés dans un milieu LA-3 inoculé avec bacilles de lépre provenant des malades de lépre ont été aussi étudiés. On n'a pas constaté un rapport entre les réponses positives stimulées par BAR cultivés et *M. leprae* de l'origine humaine; par contre, l'effet inhibitoire de tous les deux bacilles à l'incorporation de thymidine a été pareil. L'importance de l'épreuve de stimulation lymphocytaire pour l'identification des microbes est mentionnée.

## REFERENCES

1. ABE, M. A new method of indirect fluorescent antibody test for leprosy. Widely circulated memorandum of February 21, 1973.
2. ANDERSEN, V., HANSEN, N. E., KARLE, H., LIND, I., HOIBY, N. and WEEKE, B. Sequential studies of lymphocyte responsiveness and antibody formation in acute bacterial meningitis.Clin. Exp. Immunol. **26** (1976) 469-477.
3. AZUMA, T., TANIYAMA, T., SUGIMURA, K., ALADIN, A. A. and YAMAMURA, Y. Mitogenic activity of the cell walls of Mycobacteria, Nocardia, Corynebacteria and anaerobic coryniforms. Jap. J. Microbiol. **20** (1976) 263-271.
4. BARNETSON, R. StC., BJUNE, G., PEARSON, J. M. H. and KRONVALL, G. Antigenic heterogeneity of patients with reactions in borderline leprosy. Br. Med. J. **4** (1975) 435-437.
5. BJUNE, G. *In vitro* lymphocyte stimulation in leprosy; simultaneous stimulation with *Mycobacterium leprae* antigen and phytohemagglutinin. Clin. Exp. Immunol. (In press).
6. BJUNE, G. and BARNETSON, R. StC. Plasma factors in delayed-type hypersensitivity. Augmentation of lymphocyte responses in borderline leprosy reactions. Clin. Exp. Immunol. **26** (1976) 397-402.
7. BJUNE, G., BARNETSON, R. StC., RIDLEY, D. S. and KRONVALL, G. Lymphocyte transformation test in leprosy; correlation of the response with inflammation of the lesions. Clin. Exp. Immunol. **25** (1976) 85-94.
8. CLOSS, O. *In vitro* lymphocyte response to purified protein derivative, BCG and *Mycobacterium leprae* in a population not exposed to leprosy. Infect. Immun. **11** (1975) 1163-1169.
9. CLOSS, O., HARBOE, M. and WASSUM, A. M. Cross reactions between mycobacteria. I. Crossed immunoelectrophoresis of soluble antigens of *Mycobacterium lepraemurium* and comparison with BCG. Scand. J. Immunol. **4** (1975) Suppl. 2, 173-185.
10. DRAPER, P. and REES, R. J. W. A method for purification of *M. leprae* from infected armadillo tissue. Second IMMLEP Task Force Meeting, Genva, 1975.

11. GODAL, T., MYKLESTAD, B., SAMUEL, D. R. and MYRVANG, B. Characterization of the cellular immune defect in lepromatous leprosy; a specific lack of circulating *Mycobacterium leprae*-reactive lymphocytes. Clin. Exp. Immunol. **9** (1971) 821-831.

12. HALD, A. *Statistical Theory with Engineering Applications,* New York: Wiley Publications, 1952.

13. HART, P. D'ARCY and REES, R. J. W. Effect of macrocyclon in acute and chronic pulmonary tuberculosis infection in mice as shown by viable and total bacterial counts. Br. J. Exp. Pathol. **41** (1960) 414-421.

14. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo *(Dasypus novemcinctus* Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in experimentally infected armadillo. Int. J. Lepr. **39** (1971) 693-702.

15. KRONVALL, G., BJUNE, G., STANFORD, J., MENZEL, S. and SAMUEL, D. Mycobacterial antigens in antibody responses of leprosy patients. Int. J. Lepr. **43** (1975) 299-306.

16. KRONVALL, G., CLOSS, O. and BJUNE, G. Common antigen of *Mycobacterium leprae, M. lepraemurium, M. avium* and *M. fortuitum* in comparative studies using two different types of antisera. Infect. Immun. **16** (1977) 542-546.

17. RIDLEY, D. S. Reactions in leprosy. Lepr. Rev. **40** (1969) 77-81.

18. ROOK, G. A. W. The potentiating, mitogenic and inhibitory effect on lymphocytes *in vitro,* of macrophages in the lymph nodes of mice "over-loaded" with mycobacterial products. Clin. Exp. Immunol. **21** (1975) 163-172.

19. SHEPARD, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445-454.

20. SKINSNES, O. K. Problems in identifying *M. leprae.* Int. J. Lepr. **43** (1975) 267-269.

21. SKINSNES, O. K., MATSUO, E., CHANG, P. H. C. and ANDERSSON, B. *In vitro* cultivation of leprosy bacilli on hyaluronic acid-based medium. I. Preliminary report. Int. J. Lepr. **43** (1975) 193-203.

22. WORLD HEALTH ORGANIZATION. Cell-mediated immunity and resistance to infection. WHO Tech. Rep. Ser. No. 519, 1973.