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In vitro lymphocyte stimulation in leprosy; simultaneous stimulation with *Mycobacterium leprae* antigens and phytohaemagglutinin

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SUMMARY

Peripheral blood lymphocytes from 105 subjects with different forms of leprosy and healthy contacts of leprosy patients were stimulated *in vitro* with different preparations of mycobacterial antigens alone or in combination with a suboptimal dose of phytohaemagglutinin (PHA). In nearly all individuals sonicated leprosy bacilli and PHA together gave a lower ^{3}H-thymidine incorporation than did the same dose of PHA alone. There was no difference in the degree of inhibition seen in the different patient groups or the healthy contacts. High doses of whole, washed *Mycobacterium leprae*, combined with PHA led to an increased thymidine incorporation in borderline tuberculoid leprosy patients who had experienced a reversal reaction, and in healthy contacts with more than 6 months of exposure, while most lepromatous patients and contacts with less than 6 months exposure did not show an augmentation of the PHA-induced thymidine incorporation. The inhibition exerted by sonicated *M. leprae* was dose-dependent, seen even with very low doses of antigen, and was not due to direct cytotoxicity. *M. bovis*, strain BCG, was weakly suppressive in combination with PHA, and sonicated *M. duvalii* had a very marked suppressive effect. There was no correlation between the suppressive effect of *M. leprae* antigens and the other mycobacteria neither was there any correlation with the responses to the mycobacterial antigens alone. Many lepromatous leprosy patients showed significant suppression of background incorporation with addition of *M. leprae* antigens. This paper discusses whether the apparent 'non-responsiveness' in lepromatous leprosy could be due to active suppressor mechanisms operative *in vivo*.

INTRODUCTION

Antigens from *Mycobacterium leprae* can stimulate peripheral blood lymphocytes from sensitized individuals to transform and synthesize DNA *in vitro*. This is the basis for the lymphocyte stimulation test which has been utilized to establish an 'immune spectrum' in clinical leprosy (Bullock & Fasal, 1971; Godal *et al.*, 1971; Myrvang *et al.*, 1973), identify subclinical infection (Godal *et al.*, 1972a), test the influence of exposure, heredity, sex and age on the degree of sensitization (Menzel *et al.*, submitted for publication), cross-reactivity between mycobacteria (Closs, 1975), search for transfer of immunity from mother to offspring (Barnetson, Bjune & Duncan, 1976), and to study immune complications in leprosy (Godal *et al.*, 1973; Bjune *et al.*, 1976). All these studies are based on the finding of an increased DNA synthesis or morphological transformation of sensitized lymphocytes when stimulated with *M. leprae* antigens *in vitro*.

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When lymphocytes stimulated with antigen failed to incorporate more thymidine than unstimulated control cells, a lack of antigen responsive cells was considered to be the cause (Godal *et al.*, 1972b). However, it has since been noticed (Bjune, 1978) that lymphocytes from some leprosy patients incorporated less triated thymidine in the presence of antigens from *M. leprae* than control cultures with no antigen present.

As the degree of spontaneous thymidine incorporation in cultures without antigen is generally low, the inhibitory effect of *M. leprae* antigens often fails to be significant. We sought, therefore, to elevate the 'background' incorporation with subpotimal doses of phytohaemagglutinin (PHA).

In this paper we describe how the addition of *M. leprae* antigens influenced the responses to long-term stimulation with a suboptimal dose of PHA. We used peripheral blood lymphocytes from leprosy patients and healthy contacts of leprosy patients for cultivation in microtrays, and measured the responses by the incorporation of tritated thymidine. The effect of surface antigens of whole leprosy bacilli was compared to the effect of sonicated bacilli. Responses to simultaneous stimulation with PHA and *M. leprae* antigens were also compared to the responses to *M. leprae* antigens alone.

MATERIALS AND METHODS

Test subjects. A total of 155 persons were tested with three different batches of *M. leprae* antigens. 118 were patients with different clinical forms of leprosy, and thirty-seven were healthy contacts of leprosy patients.

105 of these subjects were tested with the same batch of *M. leprae* (M. 1. H-75). Twenty-seven had lepromatous leprosy (BL, LI and LL histologically) of whom five were untreated, eight had received treatment with dapsone for less than 6 months and fourteen had been treated for more than 6 months. Forty-eight were diagnosed as tuberculoid leprosy (TT, TT/BT and BT histologically). The distribution between treated and untreated cases was similar to that of the lepromatous patients. Three of the tuberculoid patients showed some inflammatory reaction in their lesions at the time of testing and nine had experienced reversal reactions within 6 months prior to the test. Thirty were healthy contacts of leprosy patients with different lengths and intensities of exposure. Lepromatous and tuberculoid leprosy patients had the same age and sex distribution and comparable socio-economic status, while the healthy contact group included mainly hospital staff with a higher socio-economic status and a lesser age variation than the patient groups. All patients were receiving medical care at the All Africa Leprosy and Rehabilitation Training Centre (ALERT) in Addis Ababa, Ethiopia.

In vitro lymphocyte stimulation test. The method has been described in detail elsewhere (Closs, 1975; Bjune *et al.*, 1976). Briefly, heparinized venous blood was drawn from the test subjects between 8.00 and 9.30 a.m. Mononuclear cells were separated by centrifugation (400 *g* for 35 min at room temperature) on Ficoll–Isopaque (spec. wt. 1·077), washed three times in ice-cold Hanks' balanced salt solution, and $1{\cdot}25 \times 10^5$ mononuclear cells were added to 200 µl volumes of Medium TC 199 supplemented with 10% pooled normal human serum, penicillin–streptomycin and glutamine. The cells were cultured in triplicates, with or without antigens and PHA, for 7 days at 37°C in 100% humidity and 5% CO_2 in air atmosphere 16 hr before harvesting, 0·5 µCi of tritiated thymidine (sp. act. 2·0 Ci/mmol) were added to each culture. The cells were harvested by suction on glass-fibre filters and the filters washed extensively in distilled water, dried and counted in a liquid scintillation counter.

Antigens. M. leprae was prepared from fresh biopsies by differential centrifugation, the details which are published elsewhere (Bjune *et al.*, 1976). The particular batch which was used for testing the 105 subjects was a pool of thirteen nodules from active lepromatous patients with a resultant morophological index (MI) of 4%. Whole washed bacilli (*M. leprae* bacilli) were used in a final concentration of 10^7 and 10^8 acid fast bacilli (AFB) per ml. Bacilli from the same batch were disrupted by ultrasonication at a concentration of 10^9 AFB/ml (sonicated *M. leprae*), and used in dilutions of 10^{-1} and 10^{-2}. Optimal doses in dose-response experiments (not shown) were within these ranges in most cases. Positive lymphocyte responses have previously been found to reach a peak on day 7, so this day was chosen as the day of harvest in these experiments.

M. bovis, strain BCG (Glaxo laboratories) was prepared by simple reconstitution in saline from freeze-dried vaccine and used in a final dilution of 10^{-1} and 10^{-2} from the concentration recommended for human vaccination (which could be counted to approximately 10^9 AFB/ml).

M. duvalii, (NCTC 358) was prepared as described previously (Kronvall *et al.*, 1975), ultrasonicated and used indilutions of 10^{-1} and 10^{-2} from a stock containing 10 mg wet weight per ml (approximately 10^9 AFB/ml).

Phytohaemagglutinin (PHA) (Reagent grade, Wellcome Research Laboratories) was used in a final concentration of 1·5 µg of protein per ml. This suboptimal dose gave a peak response on day 5, 1 day later than the optimal dose.

Statistical methods. Where not otherwise indicated, the results of stimulation are given as net counts per minute (△ ct/min): the arithmetic mean of antigen-stimulated triplicates (or triplicates stimulated simultaneously with antigen and PHA) minus the mean of triplicates without stimulants (or with PHA alone).

Significance between groups was calculated by the Kolmogorov–Smirnov two sample test. Correlations were calculated by the lesser quadrants method.

RESULTS

In some cases peripheral blood lymphocytes incorporated less ^{3}H-thymidine *in vitro* when antigens of *M. leprae* were present in the culture than when they were cultured without antigen. An example of such 'negative' responses is given in Fig. 1.

In subsequent experiments the same suboptimal dose of PHA (1·5 μg protein/ml medium) was added to all cultures. *M. leprae* antigens were added at the same time. The results revealed a striking difference in the effect of whole *M. leprae* bacilli and sonicated *M. leprae* (Fig. 2). Sonicated *M. leprae* in combination with PHA, in nearly all cases, gave a lower thymidine incorporation than in cultures with PHA alone. The suppressive effect of this antigen preparation was dose-dependent and highly significant ($P < 0{\cdot}001$). Thymidine incorporation in the presence of sonicated *M. leprae*, which was higher than the

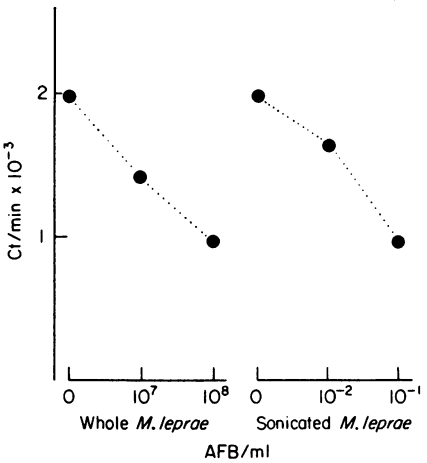

FIG. 1. Lymphocyte response in a case of untreated lepromatous leprosy measured as ^{3}H-thymidine uptake. The doses of antigen used are the same as those generally giving optimal stimulatory responses.

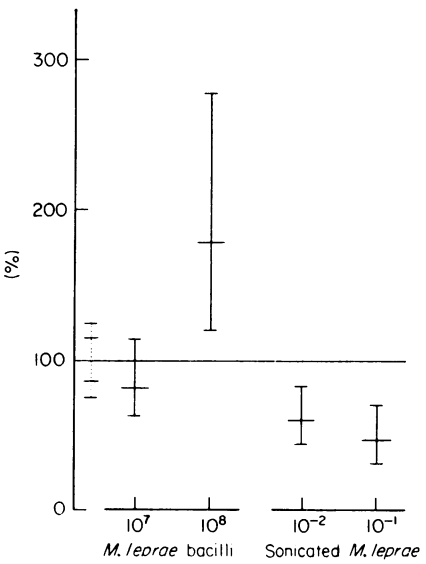

FIG. 2. Lymphocyte responses of all 105 patients and contacts to simultaneous stimulation with suboptimal doses of PHA plus *M. leprae* antigens. (— — — —) Indicates 95 and 80% confidence limits of triplicate cultures with PHA alone. The mean response of triplicates with PHA alone is, in each case, taken as 100%.

upper limit of the 95% confidence limit for the variation in cultures stimulated with PHA alone, was recorded in only four cases. All four cases were healthy contacts of leprosy patients. Contrary to the sonicate, the highest dose of *M. leprae* bacilli in combination with PHA gave rise to significantly higher thymidine incorporation than PHA alone. It is notable that individual responses to stimulation with PHA plus the higher dose of *M. leprae* bacilli showed a much greater variation than responses to PHA plus the sonicate. Three other batches of *M. leprae* antigens prepared from human biopsies by the same procedure were tested in a smaller number of leprosy patients and healthy controls. All batches showed the same clear difference between sonicate and whole bacilli in simultaneous stimulation with PHA (results not shown).

The variability of responses to simultaneous stimulation with PHA and high doses of *M. leprae* bacilli was further analysed in the 105 subjects tested with the main *M. leprae* batch (Fig. 3). Patients with lepromatous leprosy had a median response to simultaneous stimulation with PHA and the high dose of *M. leprae*, which was not significantly different from the response to PHA alone (Fig. 3a). Tuberculoid leprosy patients mainly showed an increased incorporation stimulated by this combination. Tuberculoid patients who had experienced reversal reactions within 6 months prior to testing, showed particularly high degrees of augmentation by PHA plus *M. leprae* bacilli. This increase in thymidine incorporation was much more than a simple additive effect of the response to PHA and *M. leprae* bacilli separately. Net responses to the suboptimal dose of PHA were generally in the order of 25,000 ct/min and to *M. leprae* bacilli alone, up to 3000 ct/min, while the responses to simultaneous stimulation often approached 100,000 ct/min.

Healthy contacts of leprosy patients showed, on the whole, a stimulatory effect by simultaneous

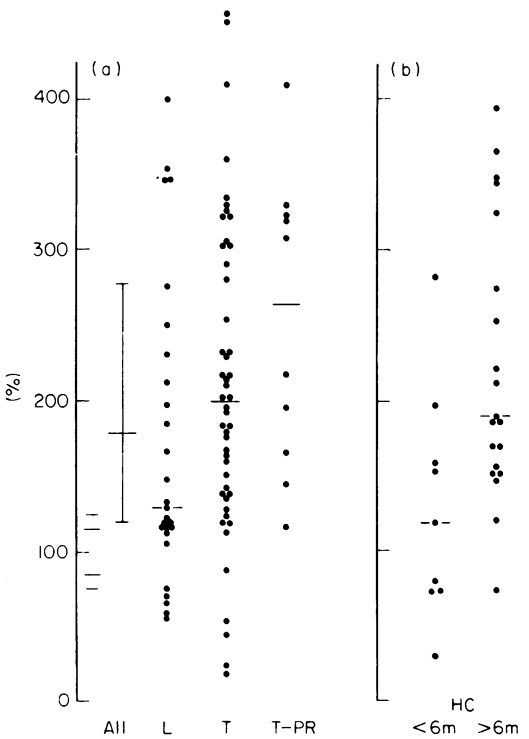

FIG. 3. (a) Lymphocyte responses to combined stimulation with PHA and 10^8 whole *M. leprae* in all 105 subjects (All), patients with lepromatous leprosy (L), patients with borderline tuberculoid leprosy (T), and borderline tuberculoid patients who had experienced a reversal reaction within 6 months before testing (T-PR). (b) The same type of responses in contacts of leprosy patients with less than 6 months of exposure (<6m) and more than 6 months exposure (>6m). (— — — —) Indicates 95 and 80% confidence interval of response to PHA alone. Mean of triplicate cultures with PHA alone is taken as 100%.

stimulation with PHA and *M. leprae* bacilli (Fig. 3b). There was, however, a clear difference between contacts with long and with short periods of exposure to leprosy patients. Contacts with less than 6 months of exposure, as a group, showed no significant increase in thymidine incorporation, beyond the incorporation in cultures with PHA alone, while contacts with more than 6 months of exposure to the bacillus showed an increased thymidine incorporation.

The suppressive effect of sonicated *M. leprae* upon incorporation in cultures simultaneously stimulated with PHA was further investigated in another group of forty leprosy patients and healthy contacts, who had a similar distribution with regard to clinical groups and length of treatment. The results are shown in Fig. 4, from which it is obvious that lower doses of sonicated *M. leprae* did not have a stimulatory effect on thymidine incorporation. The inhibitory effect of larger doses of *M. leprae* sonicate in combination with PHA is, therefore, not a result of a shift in optimal doses for stimulation towards lower doses of sonicate when it is combined with PHA. The significant inhibition by low doses of sonicate, compared to a substantial thymidine incorporation in lymphocytes cultured in the presence of even very high doses (median 10,900 ct/min with 10^8 sonicated *M. leprae* per ml), contradicts a simple toxic effect of the sonicate. The lack of direct toxicity was confirmed by the finding of similar percentages of trypan blue positive cells in cultures with high or low doses of sonicated *M. leprae* after 7 days in culture. The range of responses was small with all antigen doses tested, and there were no significant differences to be found between the responses in groups of patients or healthy contacts.

Two other mycobacterial preparations were tested in simultaneous stimulation with PHA, and in doses which usually give maximal responses when they are used as stimulants alone (Fig. 5). Both whole BCG bacilli and sonicated *M. duvalii* bacilli inhibited thymidine incorporation in combined stimulation with PHA when compared to sole stimulation with the same dose of PHA. Sonicated *M. duvalii* was the most powerful inhibitor of the two. BCG was more inhibitory than *M. leprae* bacilli, and sonicated *M. duvalii* more inhibitory than sonicated *M. leprae*. The sonicates showed a more consistent inhibitory effect and less variation in response than did the whole bacilli; this was also the case with *M. leprae* antigens. Neither of the mycobacterial preparations augmented the incorporation caused by PHA in these lepromatous patients. None of the patients responded with an increase in thymidine incorporation to the simultaneous stimulation with PHA and whole BCG, while a substantial number of lepromatous

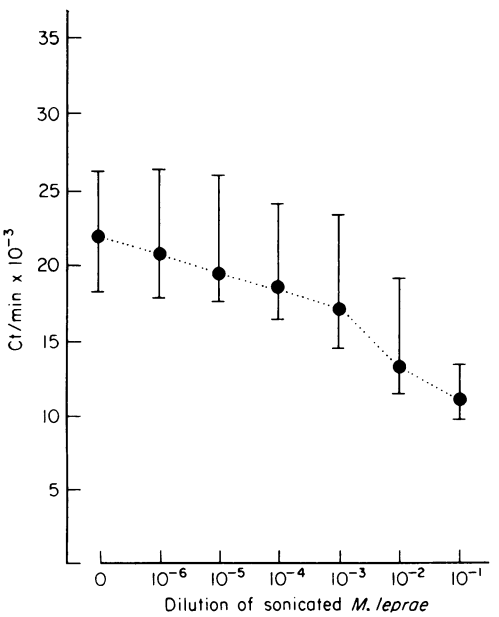

FIG. 4. Lymphocyte responses of forty individuals with different forms of leprosy and healthy contacts of leprosy to combined stimulation with sonicated *M. leprae* and PHA.

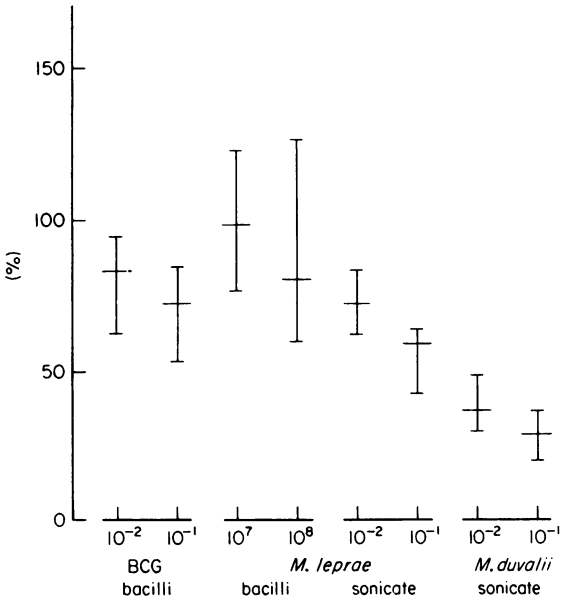

FIG. 5. Lymphocyte responses of sixteen patients with lepromatous leprosy to combined stimulation with PHA and different mycobacterial antigen preparations. The mean response to PHA alone is, in each case, taken as 100%.

individuals showed significant stimulation when tested with PHA and whole *M. leprae* simultaneously. (Fig. 5 and Fig. 3a).

Responses to *M. leprae* antigens alone were tested in the 105 patients and contacts with the main *M. leprae* batch alone, parallel to combined stimulation with antigen plus PHA. The results are shown in Fig. 6. As expected, thymidine incorporation in antigen stimulation alone was higher in tuberculoid leprosy patients than in lepromatous patients. *M. leprae* responses were even higher among healthy contacts of leprosy patients, but showed a considerable range. Some rather high responses to the high

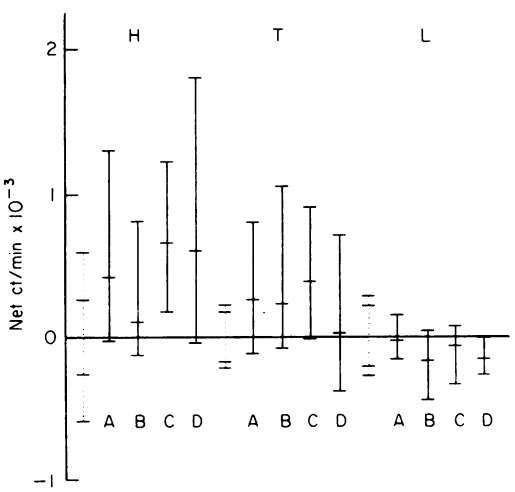

FIG. 6. Lymphocyte responses to *M. leprae* antigens in thirty healthy contacts to leprosy patients (H), forty-eight patients with borderline tuberculoid leprosy (T) and twenty-seven patients with lepromatous leprosy (BL, LI and LL) (L). (— — — —) Indicates 95 and 80% confidence limits of triplicate cultures without any stimulant added. A: whole *M. leprae* 10^{7} AFB/ml, B: whole *M. leprae* 10^{8} AFB/ml, C: sonicated *M. leprae* 10^{-2} dilution, and D: sonicated *M. leprae* 10^{-1} dilution.

dose of sonicated *M. leprae*, particularly among healthy contacts, point against a direct cytotoxic effect of this antigen preparation. Of considerable interest is the finding of several significantly suppressed responses among lepromatous leprosy patients, a group of patients which is generally considered to be completely unresponsive to *M. leprae* antigens.

Lymphocyte responses to *M. leprae* antigen alone were not correlated to the responses to *M. leprae* in combination with PHA recorded simultaneously in the same individual.

DISCUSSION

An antigen-mediated suppression of thymidine incorporation below the level in unstimulated cultures has previously been noticed in the acute phase of bacterial meningitis (Andersen *et al.*, 1976). Several microbial antigens have been shown to inhibit lymphocyte responses to PHA: *Neisseria meningitidis* (Andersen *et al.*, 1976), *Pseudomonas putida* (Hunt *et al.*, 1977), Rubella virus (Maller & Sörén, 1977), *Mycoplasma* species (Copperman & Morton, 1966), measles virus (Arstila, Herva & Ilonen, 1977), *Leishmania tropica* (Farah, Lazary & de Weck, 1975) are examples. There might be numerous causes of such inhibition. Microbial antigens can: (1) trigger macrophages to produce lymphocyte inhibitory factors (Rook, 1975), (2) stimulate the differentiation of suppressor T cells (Stobo *et al.*, 1976), (3) stimulate the differentiation of cytotoxic cells (Blanden, 1974), (4) contain cold thymidine or lead to macrophage release of cold thymidine (Opitz *et al.*, 1975), (5) be directly cytotoxic to lymphocytes (e.g. tetanus toxin), or (6) when live microbes are used as antigen, the microbe can compete with the lymphocytes for essential nutrients in the medium (Roberts, 1972).

With the exception of whole BCG bacilli, none of the antigen preparations used in this study contained live organisms, and microbial competition for essential nutrients can therefore be excluded. Trypan blue exclusion tests gave no indication of a direct cytoxic effect of mycobacterial antigens in the concentrations used in this study. The great individual differences in responses, as well as group differences according to clinical status, also makes cold thymidine or a direct cytoxic effect on lymphocytes unlikely (Fig. 6). The most likely explanation, therefore, is that mycobacterial antigens can trigger suppressor cells (Rook, 1978).

It is one of the classical dogmas in the immunology of leprosy that patients with lepromatous leprosy have no cell-mediated immune response to *M. leprae* antigens (Godal *et al.*, 1971). However, as lepromatous patients have cells which respond to *M. leprae* antigens by suppressing thymidine incorporation in non-stimulated lymphocytes, and as these patients produce large amounts of antibodies against leprosy bacilli (Estrada-Parra, 1972; Harboe *et al.*, 1978), the situation appears to be more like one of immune deviation (Asherson & Stone, 1965) than classical tolerance. The lack of delayed type hypersensitivity in immune deviation seems to be due to the specific suppression of T cell responsiveness to the antigens which have triggered off antibody production (Mackaness *et al.*, 1974). As several borderline tuberculoid patients have rather high titres of anti-*M. leprae* antibodies (Harboe *et al.*, 1978) and some patients in the same group show suppression of thymidine incorporation when stimulated with *M. leprae in vitro*, the same explanation might apply to them.

Most authors describing the inhibitory effects of microbial antigens have harvested their cultures on the day of peak responses to mitogen. We harvested our cells on the peak day for antigen responses so as to compare the results of simultaneous stimulation with PHA and antigen with responses to antigen alone. Interestingly, the inhibitory effect of rubella virus in simultaneous stimulation with PHA in mice is not evident on the peak day of responses to mitogen, but becomes clear thereafter (Maller & Sören, 1977). The influence of microbial antigen on the kinetics of lymphocyte responses to PHA might show individual variation. As the kinetics of combined stimulation were not checked individually, differences between groups of test subjects could be due to different kinetics rather than differences in lymphocyte responsiveness.

The most striking finding was the difference between the obligatory inhibitory effect of sonicated bacilli versus the highly variable, but mainly stimulatory effect of whole bacilli when lymphocytes were stimulated simultaneously with PHA and *M. leprae* antigens *in vitro* (Fig. 2). The inhibitory effect of the sonicate seems to be due not to direct cytotoxicity, as the trypan blue exclusion test revealed no

increased cell death in cultures where a high dose of sonicate was present for the 7 day incubation. Also, many cultures showed a high positive incorporation by stimulation with the sonicate alone (Fig. 6). A recently described mitogenic lectin, tridacnin (Uhlenbruck *et al.*, 1977), has an affinity for β-galactosyl groups in terminal positions. Tridacnin can inhibit competitively the effect of PHA, which indicates a similarity of receptor specificity. Arabinogalactan, a major component of mycobacterial cell walls, has a β-galactosyl in the terminal position and can thus bind to the lectin. A possible explanation for the inhibitory effect of the sonicate upon PHA-stimulated thymidine incorporation therefore is inactivation of PHA by arabinogalactan from the cell wall. If the specificity of PHA is slightly wider than for terminal β-galactosyl, it would be conceivable that the inhibition, already starting by the addition of a very low dose of the sonicate, never becomes complete (Fig. 4). The strongly inhibitory effect of sonicated *M. duvalii* (Fig. 5) could possibly be due to the same mechanism.

The augmenting effect of simultaneous stimulation with PHA and *M. leprae* bacilli could also partly be explained by arabinogalactan binding to PHA. Bacilli with a high concentration of PHA on the surface might be a more powerful stimulus to thymidine incorporation than soluble PHA in suboptimal doses. However, the group differences in responses to simultaneous stimulation indicate that a non-specific augmentation of the PHA response is not the only explanation (Fig. 3).

Stimulation of peripheral blood lymphocytes with different T cell mitogens *in vitro* can lead to the differentiation of suppressor cells which in turn have an effect on *de novo* mitogen-stimulated autologous lymphocytes (Kurnick, Bell & Gray, 1976). Products of mycobacteria, e.g. the mycolic acid–arabinogalactan–mucopeptide complex, which has a mitogenic effect on mouse lymphocytes (Azuma *et al.*, 1976) could possibly stimulate suppressor cells. The outcome of a simultaneous stimulation of lymphocytes with PHA and *M. leprae* bacilli might thus be determined by the balance between an augmented PHA stimulus offered by PHA and *M. leprae* together and the inhibitory effect of *M. leprae*-stimulating suppressor cells. The latter might not be an antigen-specific mechanism, but group differences could be due to different sizes of the suppressor population in different patient groups. We have previously discussed the possibility of control mechanisms of the immune response in leprosy patients with a chronic antigenic stimulus to their immune system (Bjune & Barnetson, 1976). In this context it is interesting to note that subjects who might be expected to have antigen available for immune stimulation, i.e. lepromatous patients, some tuberculoid patients and some contacts with a recent exposure to leprosy bacilli, show lower responses to simultaneous stimulation than subjects where antigen can be assumed to be cleared from the host, such as long-term contacts with leprosy patients and treated tuberculoid patients, particularly post-reactional ones (Fig. 3).

The lack of correlation between responses to *M. leprae* antigens alone and the same antigens in combination with PHA indicate that different mechanisms are expressed in the two test systems. A low degree of individual variation, as well as the absence of differences between groups with different clinical responses to the bacillus, indicate that the inhibition exerted by PHA and *M. leprae* sonicate in combination is a non-specific phenomenon not related to antigen-sensitized lymphocytes. The responses to combined stimulation with PHA and the higher dose of *M. leprae* bacilli might also be a non-specific phenomenon. Lack of augmentation of the PHA responses in most lepromatous leprosy patients and in some tuberculoid and short-term leprosy contacts could indicate a modulatory effect of suppressor cell activation. Antigen stimulation of suppressor cells is also a likely explanation for the finding of inhibited thymidine incorporation in lepromatous leprosy patients, in response to both antigen preparations. Antigen-sensitive suppressor cells can thus provide an alternative explanation for the clinical unresponsiveness to leprosy bacilli in this group as suggested by some workers (Turk, 1976), instead of a complete lack of *M. leprae*-sensitive T cells, as formerly assumed (Godal *et al.*, 1971).

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