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Lymphocyte response of leprosy patients to human-derived and purified armadillo-derived *Mycobacterium leprae*, BCG and PPD

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SUMMARY

The lymphocyte transformation test was applied to compare *in vitro* lymphocyte responses of tuberculoid (high resistant) and lepromatous (low resistant) leprosy patients to purified *Mycobacterium leprae* derived from experimentally infected armadillos and crude *M. leprae* derived from man, as well as to bacille Calmette–Guérin (BCG) and purified protein derivative (PPD). It was found that the purification procedure using enzymic digestion did not affect the immunogenicity of armadillo-derived *M. leprae* as compared with the crude human-derived preparation, although 2·5–5-fold higher doses of the purified organisms were required to elicitate equivalent lymphocyte responses. The result indicated the suitability of purified armadillo-derived *M. leprae* as the standard antigen for lymphocytes transformation tests in leprosy. The cross-reactivity studies show a close relationship between PPD and BCG, but not between *M. leprae* and PPD or BCG.

INTRODUCTION

Since 1971 the lymphocyte transformation test (LTT) has been extensively and successfully applied to establishing the role of the lymphocyte in the immuno-pathology of leprosy (Godal *et al.*, 1974). For the test, 'whole washed' *Mycobacterium leprae* were used as antigen and because *M. leprae* has not been cultured *in vitro*, the only adequate source of bacteria then was from biopsies of skin from advanced and previously untreated patients with lepromatous-type leprosy. However this source is limited necessitating repeated preparation of new batches and each batch requiring to be tested because not all preparations elicite lymphocyte transformation.

With the discovery by Kirchheimer & Storrs (1971) that the nine-banded armadillo (*Dasypus novemcinctus* Linn.) was highly susceptible to *Mycobacterium leprae*, tissues from this experimentally infected animal have provided an abundant source of bacteria. Furthermore, standard methods are now available for extracting and preparing high yields of purified *M. leprae* from infected armadillo tissues (see World Health Organization, 1976).

The objective of this study was to establish whether *M. leprae* derived from infected armadillo tissues could be used as a source of antigen in the LTT. This was tested by comparing purified *M. leprae* derived from infected armadillos with the cruder preparation of *M. leprae* derived from lepromatous patients, using the micro-LTT on peripheral-blood lymphocytes obtained from a series of patients with tuberculoid and lepromatous-type leprosy. Comparisons were also made between *M. leprae*, bacille Calmette–Guérin (BCG) and purified protein derivative (PPD) from *M. tuberculosis*.

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MATERIALS AND METHODS

Patients. Thirty-one patients (age range 17–64 years) suffering from leprosy were included in this study. They were classified clinically and histologically according to the Ridley–Jopling scale (Ridley & Jopling, 1966). Fourteen patients were classified as lepromatous (BL/LI, LI, LL), of which six patients were untreated. Seventeen patients were classified as tuberculoid (TT, TT/BT, BT) and all were treated. For the purpose of analysis, patients were divided into tuberculoid (high resistant) and lepromatous (low resistant) groups. No borderline (BB) and borderline lepromatous (BL) patients were included. None of the patients suffered from erythema nodosum leprosum (ENL) or reversal reaction and all patients studied were free of any other significant diseases.

Antigens. One batch of whole washed *Mycobacterium leprae* (SBR/6) was derived from a single skin nodule of an untreated patient with lepromatous leprosy. The nodule was cut into small pieces, homogenized, washed three times in human serum albumin in saline (HSA, Behringwerke AG, Marburg-Lahn) to reduce clumping of the bacteria and counted by the spot-slide method of Hart & Rees (1960). The other batch of *M. leprae* (A8) was derived from infected armadillo tissues (after exposure to 2·5 mega R ^{60}Co irradiation) of an animal inoculated intravenously with *M. leprae* from a lepromatous patient. The bacteria were extracted by enzymic digestion of the host tissue with collagenase, pronase and trypsin followed by separation from host tissues by partition in an aqueous two-phase polymer system according to the method of Draper (see World Health Organization, 1976). The acid-fast bacillary (AFB) concentrations of both batches were adjusted to 10^9/ml and stored in small aliquots at −20°C. The range of concentrations of *M. leprae* used in the LTTs were 10^6, 10^7 and 10^8 AFB/ml. BCG (Japan BCG Laboratory, Tokyo) was heat killed for 0·5 hr at 56°C and used at the same concentrations as *M. leprae* antigen and purified protein derivative, PPD (Statens Serum Institut, Copenhagen, Denmark), was used throughout the study in concentrations of 0·1, 1·0 and 10 μg/ml.

Lymphocyte transformation test. Peripheral blood was drawn into syringes containing 10 i.u. of preservative-free heparin per ml of blood. Mononuclear cells were separated on Lymphoprep (Nyegaard & Co. A/S Oslo, Norway) according to the method of Boyum (1968), washed three times with Hanks's BSS HEPES (20 mM, Flow Laboratories) and adjusted to $1{\cdot}75\times10^6$ cells/ml in culture medium (RPMI 1640, sodium bicarbonate, gentamycin 50 μg/ml and glutamine; Flow Laboratories). Each culture well (tissue-culture trays, IS–F13–96–TC, Linbro Chemical Co., New Haven, Connecticut) contained 0·2 ml cell suspension ($0{\cdot}35\times10^6$ viable cells), 25 μl of graded amounts of antigens and 25 μl homologous serum from healthy blood donors. The cultures were set up with at least nine control wells with no antigen added. Each antigen concentration was tested in triplicate. Cultures were incubated at 37°C for 7 days in an humidified atmosphere of 5% CO_2 in air. Seventy-two hr before harvest, PHA (1:100 dilution, reagent grade, Wellcome Research Laboratories, Beckenham, England) was added to three of the control wells. This was found to be a more reliable check on culture conditions and cell viability than the generally used PHA stimulation in the first 3 or 4 days. Sixteen hr before harvest, 0·5 μCi of ^{3}H-thymidine (sp. act. 2 Ci/mM, Radiochemical Centre, Amersham) was added to each well. Cells were harvested by suction on glass-fibre filters (GF/A, Whatman), washed in distilled water, air dried and counted in a liquid scintillation counter (Beckmann LS-100). The lymphocyte transformation was expressed as counts per minute (ct/min) in the cells after standard harvesting procedure. The highest mean value in the dose response curve of antigen from triplicate wells was recorded after subtracting the mean control value. A response of more than twice the control value was considered positive.

Statistical analysis. The lymphocyte response of the lepromatous and tuberculoid groups of patients to the different antigen was determined separately by linear regression analysis. Statistically significant differences in the immunogenicity of the antigens were computed by the Student's *t*-test.

RESULTS

Immunogenic correlation between Mycobacterium leprae from man and armadillo

The lymphocyte responses, expressed in ct/min, of seventeen tuberculoid patients show a close correlation ($r = 0{\cdot}978$; $P<0{\cdot}001$) between purified *M. leprae* from armadillo (A8) and human-derived *M. leprae* (SBR/6) (Fig. 1a). Ten of the patients show positive lymphocyte responses to *M. leprae* including two very strong responders, to both antigens. The two very strong responders had burnt-out borderline-tuberculoid (BT) leprosy. The arithmetic mean value of the lymphocyte responses to SBR/6 was 1·75 higher than that to A8, both antigens being used at the same concentrations. In the lepromatous group of fourteen patients no positive response (less than two times the control value) to either *M. leprae* antigen was observed. In fact their responses were mostly lower than the control values, explaining the negative values in Fig. 1b. The non-responsiveness of lymphocytes of lepromatous patients to both *M. leprae* antigens also indicated similar immunogenic characteristics shared by SBR/6 and A8. A significant correlation was found between SRB/6 and A8 ($0{\cdot}001<P<0{\cdot}01$; $r = 0{\cdot}67$).

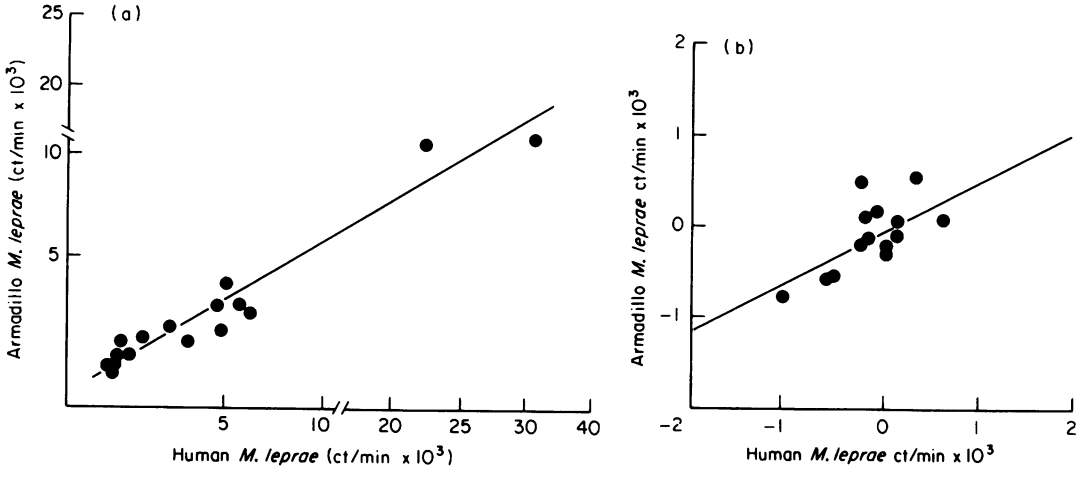

FIG. 1. *In vitro* transformation of lymphocytes from tuberculoid patients (a) and lepromatous patients (b) to human-derived (SBR/6) and purified armadillo-derived *Mycobacterium leprae* (A8). Ct/min = count per min of ^{3}H-thymidine incorporation by the stimulated culture minus cpm in control culture. (a) $r = 0{\cdot}978$, $P < 0{\cdot}0001$. (b) $r = 0{\cdot}67$, $P < 0{\cdot}01$.

Dose-response relationship between M. leprae from man and armadillo

In the results reported above, the lymphocyte responses of tuberculoid patients to SBR/6 were significantly higher than those to A8 at the same bacillary concentration per culture. This is true for each responding patient tested. To determine whether there was a qualitative as well as a quantitative difference in the immunogenicity of the two antigen preparations, lymphocytes from five of the tuberculoid patients were tested against SBR/6 and A8 at the same bacillary concentrations (10^8AFB/ml) as well as against A8 at $2{\cdot}5$ and $5{\cdot}0 \times 10^8$AFB/ml. The pattern of responses (Fig. 2) are consistent with an entirely quantitative difference, since the lymphocyte responses to A8 increased when higher concentrations of antigen were used. From earlier studies it was shown that a concentration of 10^8AFB/ml of whole human-derived *M. leprae* (SBR/6) was optimal for the stimulation of lymphocytes. The

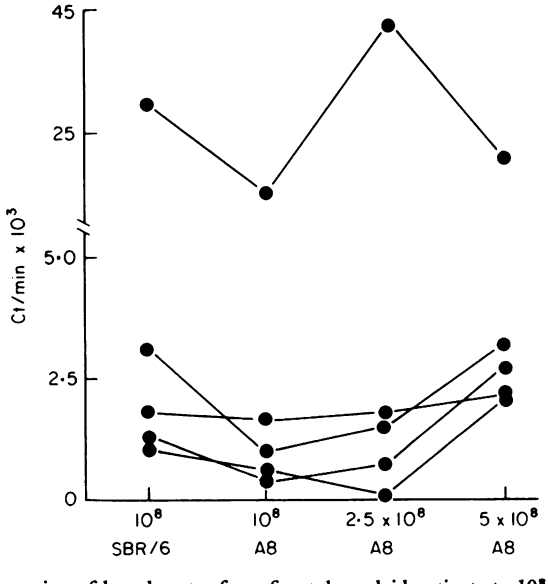

FIG. 2. *In vitro* transformation of lymphocytes from five tuberculoid patients to 10^8 human-derived *Mycobacterium leprae* (SRB/6) and 10^8, $2{\cdot}5 \times 10^8$ and $5{\cdot}0 \times 10^8$ armadillo-derived *M. lepare* (A8). Ct/min, see Fig. 1.

present data showed that some 2·5–5 times higher concentrations of purified armadillo-derived AFB (A8) were required to elicit a level of lymphocyte stimulation equivalent to that produced by 10^8AFB/ml of SBR/6.

Comparison between human-derived M. leprae, PPD and BCG

In the tuberculoid group of patients there was no correlation between the lymphocyte responses to BCG and *M. leprae* ($r = 0{\cdot}39$) and between PPD and *M. leprae* ($r = 0{\cdot}17$) (Fig. 3a, 3b). Thus, while nine out of ten patients tested who responded to *M. leprae* also responded to BCG, of the seven patients who did not respond to *M. leprae*, four did not respond to BCG and three responded weakly to BCG.

The lymphocytes of lepromatous patients remained non-responsive to SBR/6 throughout. However,

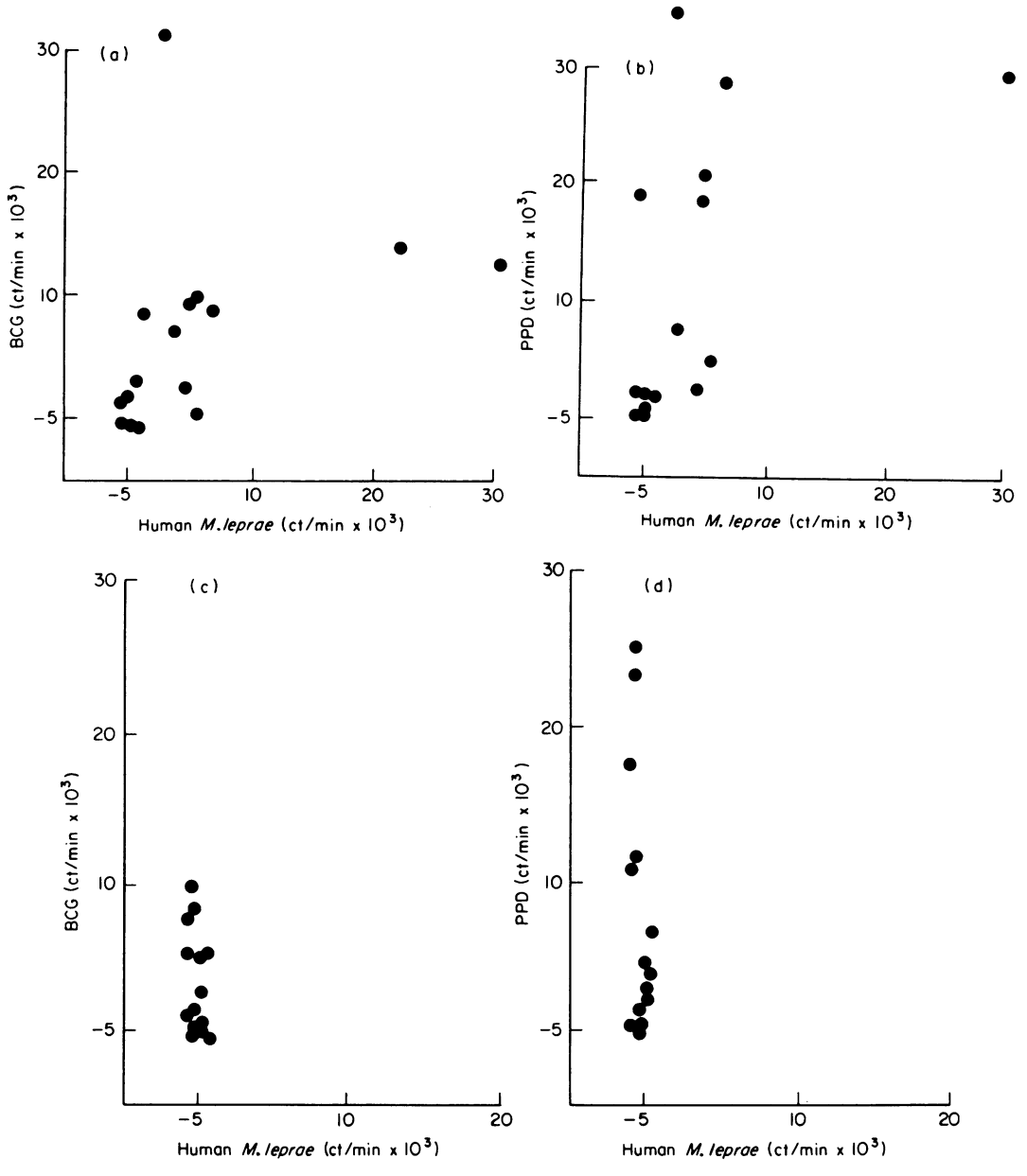

FIG. 3a–d. For legend see p. 168.

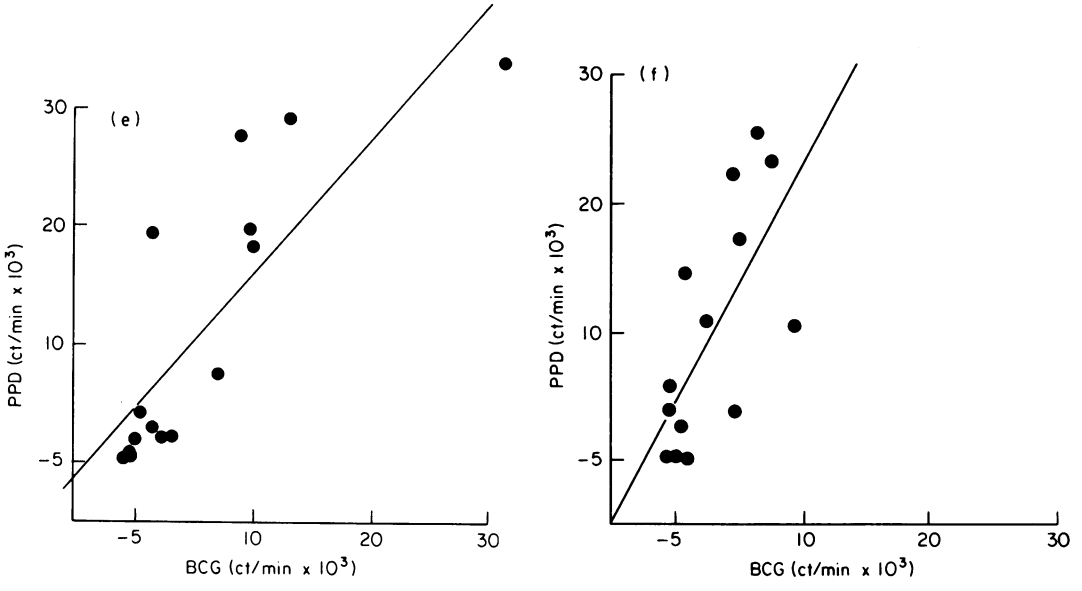

FIG. 3. Comparison of *in vitro* transformation of lymphocytes from (a) seventeen tuberculoid patients to SBR/6 and BCG, ($r = 0{\cdot}39$); (b) fifteen tuberculoid patients to SBR/6 and PPD, ($r = 0{\cdot}17$); (c) fourteen lepromatous patients to SBR/6 and BCG; (d) fourteen lepromatous patients to SBR/6 and PPD; (e) fifteen tuberculoid patients to BCG and PPD ($r = 0{\cdot}82$, $P < 0{\cdot}001$) and (f) seventeen lepromatous patients to BCG and PPD, ($r = 0{\cdot}71$, $P < 0{\cdot}01$). Ct/min, see Fig. 1.

seven patients showed positive responses to BCG and eleven to PPD (Fig. 3c, 3d). On the other hand a strong correlation was found to exist between whole BCG and PPD in LTTs in tuberculoid ($P < 0{\cdot}001$; $r = 0{\cdot}82$) as well as lepromatous patients ($0{\cdot}001 < P < 0{\cdot}01$; $r = 0{\cdot}71$) (Fig. 3e, 3f).

DISCUSSION

The LTT has been successfully applied to characterize the specific cell-mediated defect in lepromatous leprosy (Godal *et al.*, 1971), to define the immunological spectrum in leprosy (Myrvang *et al.*, 1974), and was considered as an *in vitro* correlate of delayed type hypersensitivity to mycobacterial antigens (Bjune *et al.*, 1976). In the present study, the LTT was used to compare the responsiveness of lymphocytes from a range of patients with leprosy to different preparations of *Mycobacterium leprae* and other species of mycobacteria, in order to determine the sensitivity and specificity of the antigen component of the test.

The first comparison with this test system showed that a preparation of purified armadillo-derived *M. leprae* (A8) was immunogenically similar to a crude preparation of human-derived organisms (SBR/6). Thus the qualitative responsiveness of lymphocytes from tuberculoid patients was identical to both preparations. Similarly, lymphocytes from lepromatous patients, known to be unresponsive in LTTs to human-derived *M. leprae*, remained unresponsive throughout to purified bacilli from armadillos. However, the purification procedures apparently had quantitatively reduced the immunogenicity in tuberculoid patients because lymphocyte stimulation values were significantly lower for A8 than for SBR/6. Thus up to five-times-higher concentrations of A8 were required to give equal stimulation values as obtained with SBR/6. These quantitative differences in the LTT are consistent with the comparative studies on these two preparations as skin-test lepromins, where at least a five-times-higher concentration of purified bacilli are required to elicit equivalent skin reactions (Rees & Desikan, unpublished data). In spite of these quantitative differences, which are likely to be overcome by modifying the procedures of purification, these findings indicate the importance of using purified armadillo-derived

M. leprae as the antigen of choice for standardizing the important *in vitro* (LT) and *in vivo* (Mitsuda) tests in leprosy.

Cross-reactivity between whole BCG and soluble PPD antigens in lymphocyte cultures of healthy individuals has been reported by Closs (1975). The present study has confirmed this finding in leprosy patients. On the other hand, the immunogenic analysis in the present system has indicated that there is little cross-reactivity between *M. leprae* and BCG or PPD. The correlation patterns of lymphocyte responses of lepromatous and of tuberculoid patients are rather similar, indicating that lymphocytes of both groups do respond to the same antigens. Thus if BCG or PPD and *M. leprae* share various antigens, one would expect, in contrast to our data, lymphocytes of lepromatous patients, not recognizing the *M. leprae* related antigens, to respond less to these antigens. One would also expect a better correlation between BCG or PPD and *M. leprae* in the tuberculoid group. Thus Closs's finding of cross-reactivity between *M. leprae* and BCG in healthy people cannot be confirmed in leprosy patients.

An important criterion for studying the immunogenic relationship of *M. leprae* and other mycobacterial species is the inability of T cells of lepramatous patients to respond to the leprosy bacillus. However, we found that lymphocytes of lepromatous patients often responded well to *M. nonchromogenicum* and two strains of *M. vaccae* (unpublished data), although these mycobacteria have been found to be antigenically closely related to *M. leprae* in immunodiffusion analysis (Stanford *et al.*, 1975). This apparent discrepancy emphasizes the difference between antigenicity and immunogenicity of an antigen. It may also indicate the relative sensitivity of the LTT and immunodiffusion analysis. Hitherto *M. leprae* is the only known mycobacterium to which lymphocytes of lepromatous patients do not respond *in vitro*, underlining one of the unique features of the leprosy bacillus.

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