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Effects of *Mycobacterium leprae* antigens on the *in vitro* responsiveness of mononuclear cells from armadillos to Concanavalin-A

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Summary Armadillos, immunologically intact animals, develop a disseminated disease analogous to lepromatous leprosy in man when experimentally infected with *Mycobacterium leprae* of human origin. However, some animals show resistance to such infection. In this experiment the suppressive effect of *M. leprae* on Con-A-induced *in vitro* proliferation of mononuclear cells, drawn from both susceptible and resistant armadillos, is investigated.

Peripheral blood mononuclear cells from armadillos which were resistant to infection with *M. leprae* consistently showed suppressed responses to Con-A when concomitantly exposed to antigens of *M. leprae*. Armadillos with disseminated *M. leprae* infections had mononuclear cells which responded to *M. leprae* by suppressing responses to intermediate doses of Con-A, enhancing the response to an optimal dose of Con-A, and inducing no significant change in response to a minimal dose of Con-A.

These observations in armadillos support the reports in human leprosy studies that *M. leprae*-induced suppression of a Con-A response is associated with resistance.

Human leprosy is a spectral disease where paucibacillary tuberculoid leprosy, and multibacillary lepromatous leprosy are associated with high and low resistance respectively. Polar lepromatous leprosy (LL) is characterized by the nondetectability of cell-mediated immunity to the antigens of *Mycobacterium leprae*. Both *in vivo* and *in vitro* studies strongly implicate a T-cell associated defect in LL patients; however, the precise nature of this antigen specific T-cell deficiency is unknown. In recent years several laboratories have investigated the role of suppressor T-cells in leprosy patients. Using various *in vitro* functional studies, reports have shown that *M. leprae*-induced suppression of a mitogen or antigen response is associated with patients with potential resistance (tuberculoid)[18, 23] in individuals with presumably effective immunity (lepromin positive normal individuals),[23] and in occupationally exposed and presumably subclinically

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infected healthy subjects.[24] *M. leprae*-induced suppression of a Concanavalin-A (Con-A) mitogen response was conspicuously absent in disseminated multibacillary lepromatous leprosy.[18,23] It was further shown using co-cultures of lymphocytes from HLA-D matched siblings that hyperactive clones of suppressor cells were not present in lepromatous patients.[19,25] On the other hand, results from a series of elegant studies indicate that *M. leprae*-induced suppression of a Con-A response is associated with TH_2^+ cells.[16] TH_2^+ cells which suppress in the presence lepromin were demonstrated in most American and Venezuelan patients with lepromatous leprosy, but were absent in tuberculoid patients or normal controls.[15]

Clearly there are distinct differences in experimental results using co-culture techniques with Con-A and *M. leprae* antigens to test for suppressor cell phenomena in human leprosy. In view of these conflicting data, we considered it relevant to use an animal model and avoid some of the inherent variabilities in the investigation of human disease.

Armadillos (*Dasypus novemcinctus*, Linn.) are immunologically intact animals which (a) develop a disseminated infection with *M. leprae*,[11] (b) display marked differences in susceptibility to leprosy,[12] (c) have histopathological evidence suggestive of indeterminate leprosy in man[13] and responses showing both reversal and erythema nodosum leprosum reactions.[9] Although not bred under controlled conditions, armadillos from a given locale and housed after captivity in a uniform environment might be expected to be relatively uniform in their exposure to environmental mycobacteria. For experimental purposes, they have the advantages over humans with the disease in that they have a known, quantitative exposure to *M. leprae* and they can be studied in the absence of drugs. These animals, therefore, seem to offer a suitable model in which to test the hypothesis that suppressor cells are associated with susceptibility to disseminated leprosy.

Using the *in vitro* system advocated by Mehra *et al.*[15,16] to demonstrate *M. leprae* specific suppressor cells in peripheral blood, we have studied armadillos with disseminated leprosy and compared them with armadillos from the same colony which had demonstrated resistance to the disease. In this system, ^{3}H-thymidine incorporation is measured in replicate sets of mononuclear cell cultures, one exposed to Con-A alone and one exposed to Con-A plus sonicated Dharmendra lepromin. A reduction in ^{3}H-thymidine incorporation in the cultures containing sonicated Dharmendra lepromin is interpreted as meaning that *M. leprae* has specifically triggered suppressor cells with non-specific suppressor effects.

Sonicated Dharmendra lepromin consistently suppressed the *in vitro* mononuclear cell responses to Con-A in armadillos which have shown resistance to infection with leprosy bacilli. In armadillos with disseminated *M. leprae* infections, sonicated Dharmendra lepromin had mixed effects on the response of mononuclear cells to Con-A. These effects varied with the concentration of Con-A used *in vitro*.

The present investigation in armadillos supports reports that *M. leprae* induced suppression of mitogen responses is associated with the development of resistance.

Materials and methods

ARMADILLOS

Armadillos were purchased from local trappers and were all captured within a 30-mile radius of Carville Louisiana. They had been housed at Carville for at least 1 year at the time they were studied. Husbandry procedures have been described.[26]

Eighteen armadillos were designated as susceptible to leprosy and were defined as those having a systemic infection with *M. leprae*. At the time of study, these animals had received IV injections of 10^7 to 10^8 viable *M. leprae* 9–12 months earlier. They harboured $3{\cdot}06\ (\pm 2{\cdot}09) \times 10^9$ (mean $\pm$ SD) *M. leprae* per g liver, $1{\cdot}13\ (\pm 7{\cdot}02) \times 10^9$ per g spleen and $3{\cdot}26\ (\pm 2{\cdot}21) \times 10^{10}$ per g of lymph node tissue. The bacilli from these animals oxidized D-dopa,[22] and their acid-fastness was lost after pyridine treatment.[17] Each of the susceptible animals was tested once at the time of sacrifice for harvesting *M. leprae*.

Five armadillos were designated as resistant to leprosy, and were defined as healthy, long-term survivors of challenge inoculations with viable *M. leprae*. Three (Nos 199, 321 and 323) had been vaccinated with 3×10^8 heat-killed *M. leprae* in incomplete Freund's adjuvant. Armadillo No. 199 had been challenged with 10^6 viable bacilli and Nos 321 and 323 challenged with 6×10^3 viable bacilli. At the time of study, armadillo No. 199 was apparently healthy 7 years after challenge and Nos 321 and 323 were apparently healthy 3 years after challenge. The other two resistant animals were not vaccinated. One of these, armadillo No. 40, has survived in apparent good health for 10 years after being inoculated intradermally with 2×10^8 viable *M. leprae*. Four other animals receiving the same inoculum had succumbed to systemic infection with *M. leprae* 1–3 years after inoculation. The other, No. 301, was one of five apparently healthy animals 5 years after intradermal inoculation of a group of 19 with 10^4 viable *M. leprae*. The remaining 14 animals in this group developed disseminated leprosy 1–5 years after inoculation. Armadillo No. 199 was tested three times, No. 321 twice, No. 323 four times, No. 40 twice and No. 301 twice.

Mitogens and antigen(s)

1 Concanavalin-A (Con-A)—(highly purified Type IV, Sigma Chemical Co., St Louis, MO, Lot No. 58C-7700).
2 Dharmendra Antigen(s).

Acid-fast bacilli were harvested from 12 g of infected armadillo spleen tissue containing 2×10^{10} AFB/g (Lot A) and 12 g of infected armadillo lymph node containing 1×10^{11} AFB/g of tissue (Lot B). These tissues were negative for bacterial growth on fluid thioglycollate (DIFCO Laboratories, Detroit, Michigan), Trypticase™ Soy Broth and Mycobactosel™ L-J Medium Slants (BBL, Cockeysville, MD). The non-cultivatable acid-fast bacilli were isolated from the infected tissues by the method of Prabhakaran *et al.*[21] using density gradient centrifugation of homogenized tissues on solutions of sucrose and KCL. The isolated bacilli were then used to prepare antigen using a slightly modified method of Dharmendra.[6] The *M. leprae* isolate Lot A containing a total of $1{\cdot}4 \times 10^{11}$ AFB (58% yield) and Lot B containing $6{\cdot}6 \times 10^{11}$ AFB (55% yield) were added to chloroform, and ground using a mortar and pestle. The complete evaporation of chloroform was followed by the addition of 10 ml ether. The AFB-ether mixture was centrifuged at $26{,}500 \times \boldsymbol{g}$ for 30 min at 4°C (Sorvall RC 2-B centrifuge fitted with SS-34 head, Dupont Instruments, Newton, Conn.) and the supernatant discarded. The sediment was washed again with 8 ml ether and the ether was allowed to completely evaporate from the sediment at room temperature. The dry sediment was suspended in physiological saline and autoclaved. The autoclaved suspensions were sonicated at 4°C for 10 min at 50 watts (Sonifier Cell Disruptor, Model W-185, Heat Systems Ultrasonics, Inc., Plainsview, NY) and the concentration of protein was determined by the method of Lowry *et al.*[14] using bovine serum albumin as a standard. Thirteen millilitres from Lot A contained 350 μg of protein/ml and 17·0 ml from Lot B contained 1300 μg of protein/ml. Both lots were titrated for dose responsiveness, and toxicity to mononuclear cells.

Preparation of mononuclear cell suspension

Blood was collected in heparinized vacuum tubes by cardiac puncture immediately prior to sacrificing the susceptible animals and by venipuncture of the resistant ones. A sample was removed for white blood cell (WBC) enumeration (Coulter Counter, Coulter Electronic Inc., Hialeah, FL) and differential counts after Wright-Giemsa staining. Mononuclear cells were then isolated by discontinuous density gradient centrifugation. Twenty millilitres of a 1:1 mixture of heparinized whole blood and physiological saline was layered onto 6·0 ml of Ficoll–Hypaque (Lymphoprep®, Nyegaard and Co., Oslo, Norway) and centrifuged at $400 \times \boldsymbol{g}$ for 45 min at 20°C. The mononuclear cells isolated from the Ficoll–Hypaque-plasma interface were resuspended with physiological saline to a volume of 40 ml and centrifuged at $800 \times \boldsymbol{g}$ for 15 min at 20°C. To deplete platelets, the mononuclear cells were then resuspended with saline to 40 ml and centrifuged at $250 \times \boldsymbol{g}$ for 10 min. The supernatant was discarded and the pelleted mononuclear cells were then resuspended in 3·0 ml of RPMI 1640 (Associated Biomedics Systems, Buffalo, NY), a WBC performed, and the percentages of lymphocytes and viable cells determined by the leukocyte peroxidase stain[10] and

exclusion of 0·4% w/v trypan blue in normal saline respectively. The mononuclear cell suspension was diluted to contain a final concentration of 1×10^6 viable, peroxidase negative cells per ml in RPMI-1640 supplemented to contain 20% v/v heat inactivated fetal calf serum (Pacific Biological, Bio-Rad Laboratories, Richmond, CA), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Cell cultures, labelling and harvesting

Peroxidase negative, viable cells (2×10^5) were incubated for 72 hr in U-bottom polystyrene microtitration plates (Dynatech Laboratories, Inc., Alexandria, VA) in a final volume of 200 μl. The cells were exposed for various time periods to the following antigen(s) and mitogen:

1 Con-A was added in concentrations of 3·12, 0·78 and 0·19 μg per well and the cells in 4–5 replicate cultures were harvested after 72 hr of incubation.
2 Con-A plus Dharmendra antigen(s): Con-A was added in concentrations of 3·12, 0·78 and 0·19 μg per well together with a constant amount of sonicated Dharmendra antigen(s) (15 μg protein per well). The cells in 4–5 replicate cultures were harvested after 72-hr incubation.
3 Sonicated Dharmendra antigen(s): Sonicated Dharmendra antigen(s) (15 μg protein per well) was added and the cells in 6–8 replicate cultures were harvested after 3 and 7 days of incubation.

The cultures were incubated at 37°C in a 5% CO_2–95% air atmosphere with a relative humidity greater than 95%. The mitogen and antigen plus mitogen cultures were pulsed for 5–6 hr with 1 μCi of tritiated thymidine (^{3}H-thymidine, specific activity 71·8 Ci/mM, New England Nuclear, Boston MA) in 10 μl of saline. The cultures were subsequently harvested onto fibreglass filters using a mechanical cell harvester (Titertek, Flow Laboratories, Inc., McLean, VA). One hundred millilitres of NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, IL) were added to moist filters in scintillation vials. After 15 min at room temperature, 6·0 ml of scintillation counting fluid (Eastman Ready-to-Use I) was added. The vials were placed into a Beckman LS-250 liquid scintillation counter for counting. The incorporation of ^{3}H-thymidine was corrected for background and quenching and results were expressed as disintegrations per minute (DPM). Results were analysed for statistical significance on a Hewlett Packard 9845B computer using the one-tailed paired *t*-test and by its non-parametric equivalent, the Wilcoxon signed rank test.[4]

Results

Preliminary experiments involved the preparation and characterization of sonicated Dharmendra antigen(s) and establishing dose–response relationships

of Con-A to determine minimal, intermediate, and optimal doses of the mitogen. To determine the degree of specificity and toxicity of sonicated Dharmendra antigen(s) Lots A and B were titrated in microculture using mononuclear cells from a healthy human contact of leprosy patients (A.B.) and a presumed leprosy naïve individual (F.B.). The results of this titration are represented in Table 1. In both Lots A and B, 15 μg of sonicated Dharmendra antigen(s) per well was optimal. After titration, these lots were pooled, aliquoted and stored at −80°C. Only the required amount was thawed as needed for each experiment.

A Con-A dose–blastogenic response relationship with mononuclear leuko-

Table 1. Six-day blastogenic response of mononuclear cells from healthy humans exposed to various concentrations of integral *M. leprae* and Lots A and B preparation of sonicated Dharmendra antigen(s)

Culture + antigen(s)	N*	A.B.†	SI‡	N	F.B.§	SI
None (control)	8	668·4 ± 184·5¶	1	8	979·7 ± 298·9	1
Integral *M. leprae*						
100:1**	4	1497·9 ± 73·3	2·2	4	399·8 ± 29·7	0·4
10:1	4	1162·8 ± 297·4	1·7	4	Not done	
1:1	4	433·4 ± 257·1	0·6	4	308·9 ± 23·5	0·3
Dharmendra Lot A						
30‡‡	4	2942·5 ± 806·5	4·4	3	1183·9 ± 274·3	1·2
15	4	5915·3 ± 1111·5	8·8	3	1009·4 ± 369·3	1·0
3	4	4247·9 ± 1458·0	6·4		Not done	
0·6	4	2998·2 ± 618·5	4·5	3	543·7 ± 40·7	0·6
0·12	4	1179·6 ± 481·9	1·8		Not done	
0·024	4	1003·6 ± 254·6	1·5	3	473·0 ± 28·6	0·5
Dharmendra Lot B						
30	3	3656·2 ± 1379·0	5·5	2	742·0 ± 188·6	0·8
15	4	5106·5 ± 1253·6	7·6	2	539·8 ± 68·7	0·6
3	4	3148·3 ± 122·5	4·7	3	696·6 ± 127·5	0·7

* Number of microtitre wells assayed.

† A.B. = individual in frequent contact with leprosy patients and previously titrated as a high responder to *M. leprae*.

‡ Stimulation Index $= \frac{\text{Culture + Antigen}}{\text{Control}}$.

§ F.B. = assumed leprosy naïve individual.

¶ Mean ± SD DPM's.

** Ratio of integral *M. leprae* organisms to viable lymphocytes in culture.

‡‡ Concentration of sonicated Dharmendra antigen in μg of protein/well.

cytes isolated from the peripheral blood of three normal armadillos was determined after 76-hr exposure to varying concentrations of Con-A. (Data not shown.) Doses of 0·04 μg per well were not stimulatory; 0·19 μg caused minimal stimulation and 0·78 μg moderate stimulation. The optimal dose of Con-A, with respect to ^{3}H-thymidine incorporation, was 3·125 μg per well; 6·25 μg/culture was slightly inhibitory and 12·5 μg was strongly inhibitory when compared to the response at 3·125 μg per well. For lymphocyte blastogenesis in armadillos Con-A was used at 3·125 μg as a maximum stimulatory concentration, 0·78 μg as an intermediate or mid-range stimulatory concentration and 0·19 μg as a minimal or low stimulatory concentration.

Results of the dose responsiveness of mononuclear cell cultures from resistant and susceptible armadillos to Con-A alone and the effects of the addition of sonicated Dharmendra lepromin in co-culture with Con-A are shown in Table 2. The mononuclear cell cultures from susceptible and resistant armadillos were similar, each containing 3·35 ($\pm$0·5) $\times 10^5$ (mean $\pm$ SD) and 3·32 ($\pm$1·2) $\times 10^5$

Table 2. Incorporation of ^{3}H-thymidine by armadillo peripheral blood mononuclear cells stimulated with Con-A alone or stimulated with Con-A plus sonicated Dharmendra (S. Dharmendra) lepromin

Culture	Source of cultured mononuclear cells: Susceptible armadillos (N = 18)	Source of cultured mononuclear cells: Resistant armadillos (N = 13)*
Controls	1209 ± 329†	1051 ± 256
Con-A (0·19 μg)	4518 ± 1971	5609 ± 1973
Con-A (0·19 μg) + S. Dharmendra	3771 ± 1520	2157 ± 683§
% Suppression by S. Dharmendra	16·5‡	61·5
Con-A (0·78 μg)	40,668 ± 10,064	40,192 ± 11,677
Con-A (0·78 μg) + S. Dharmendra	21,858 ± 11,642¶	16,099 ± 6688¶
% Suppression by S. Dharmendra	46·3	59·9
Con-A (3·125 μg)	113,205 ± 26,790	162,896 ± 43,650
Con-A (3·125 μg) + S. Dharmendra	153,014 ± 34,111¶	101,852 ± 34,250¶
% Suppression by S. Dharmendra	−35·2	37·5

* Multiple assays were done on each of five resistant armadillos (No. 40, N = 2; No. 199, N = 3; No. 301, N = 2; No. 321, N = 2; No. 323, N = 4).

† Mean values were calculated in each experiment based on four to five replicate cultures. These mean values were used in calculating the presented means ± SEM DPM/well.

‡ % Suppression by sonicated Dharmendra = 100 − [(DPM of Con-A + sonicated Dharmendra × 100 ÷ DPM of Con-A]

§ Significantly different from Con-A alone, p < 0·05, paired *t*-test or Wilcoxon signed rank test.

¶ Significantly different from Con-A alone, $p < 0·01$, paired *t*-test or Wilcoxon signed rank test.

mononuclear cells respectively. The monocytes (peroxidase positive cells) in culture averaged ±SD 1·35 (±0·5) × 10^5 for susceptible armadillos and 1·32 (±1·2) × 10^5 for resistant armadillos. The percentages of viable cells averaged 98% (ranging from 95 to 100%) for both groups of armadillos.

There was no significant difference between the responses to Con-A alone of mononuclear cell cultures from susceptible and those of resistant armadillos. The

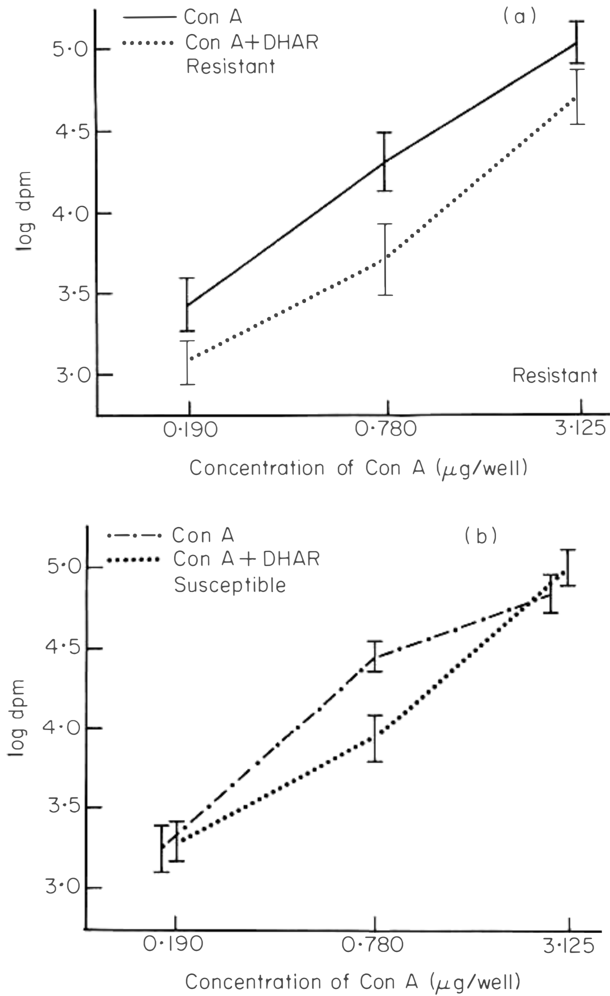

Figure 1(a). Mean ±SEM DPM ^{3}H-thymidine incorporation of peripheral blood mononuclear cells from leprosy susceptible (S) armadillos after exposure for 72 hr to Concanavalin-A (Con-A) alone or concomitantly with 15 μg of sonicated Dharmendra antigen (DHAR). (b) Mean ±SEM DPM ^{3}H-thymidine incorporation of peripheral blood mononuclear cells from leprosy-resistant (R) armadillos after exposure for 72 hr to Concanavalin-A (Con-A) alone or concomitantly with 15 μg of sonicated Dharmendra antigen (DHAR).

simultaneous addition of Con-A and sonicated Dharmendra lepromin had varying effects when compared to cultures receiving Con-A alone. Susceptible armadillos responded to sonicated Dharmendra lepromin by suppressing responses to intermediate doses of Con-A, enhancing responses to the highest dose of Con-A, and showing no significant change in response to the lowest concentration of Con-A. Mononuclear cell cultures from resistant armadillos responded to sonicated Dharmendra lepromin by significantly suppressing responses to all three concentrations of Con-A.

Examining the data graphically (Figure 1(a) and (b)) it may be seen that there is a consistent depression by sonicated Dharmendra lepromin in resistant animals and that the dose–response relationships of Con-A with and without sonicated Dharmendra lepromin are essentially parallel (Figure 1(a)). In susceptible armadillos, sonicated Dharmendra lepromin causes a change in the dose–response relationship resulting in no change at the low dose, suppression at the intermediate dose and enhancement of the response at the high dose of Con-A (Figure 1(b)).

Sonicated Dharmendra lepromin alone was added to other mononuclear cell cultures from these animals. In 3-day cultures there was a statistically significant ($p < 0{\cdot}025$ one-tailed paired t-test or Wilcoxon signed rank) blastogenic response in susceptible armadillos (1595 ± 416 [15], mean ± SEM [N] DPM in control, unstimulated cultures vs. 4188 ± 1573 in replicate, Dharmendra-stimulated cultures) but in resistant animals (902 ± 341 [7] in control cultures vs. 7664 ± 2897 in replicate, Dharmendra-stimulated cultures) the differences were not significant. In 7-day mononuclear cell cultures, neither the susceptible (882 ± 365 [16] in controls vs. 1360 ± 729 in replicate Dharmendra-stimulated cultures) or the resistant (450 ± 100 [13] in controls vs. 1946 ± 861 in replicate, Dharmendra-stimulated cultures) armadillos showed statistically significant stimulation by Dharmendra lepromin.

Discussion

Studies of Mehra[16, 17] have utilized a single concentration of Con-A which is given as 0·4 or 0·5 μg/well. The human Dharmendra antigen was prepared by Abe (National Institute for Leprosy Research, Tokyo, Japan) and is represented in cultures as ‘1:10’. [In our laboratory an aliquot from this same lot of Dharmendra antigen contained $2{\cdot}9 \times 10^6$ acid-fast bacilli ml^{-1} and 826 μg of protein ml^{-1}.] With these reagents, Mehra *et al.*[16] have shown that peripheral blood mononuclear cell cultures from some human leprosy patients show lower incorporation of ^{3}H-thymidine when exposed to both Con-A and Dharmendra lepromin than if stimulated with Con-A alone. This has been interpreted by these investigators as detecting *M. leprae* specific suppressor cells in these individuals. By these criteria, these cells were found in 32 of 35 lepromatous and 15 of 15

borderline leprosy patients, but in only 2 of 15 tuberculoid patients and 2 of 30 healthy control subjects. Later these cells were shown to belong to the TH_2^+ subset of T lymphocytes.[16]

Quite to the contrary, Nath & Singh[18] using whole, autoclaved *M. leprae* in co-culture with an optimally stimulatory concentration of Con-A, describe an antigen-generated suppression of Con-A stimulated lymphocyte transformation in 80% (17/21) of tuberculoid leprosy patients. In lepromatous patients, they describe variable results but the number of patients showing suppression was low and generally the responses were enhanced when *M. leprae* was added to lymphocyte cultures stimulated with Con-A.

The present findings in armadillos using 15 μg sonicated Dharmendra antigen prepared from armadillo grown *M. leprae*, and earlier work in humans with leprosy using 15–30 μg of Dharmendra antigen prepared by Abe, are in agreement with Nath & Singh[18]; in that the Dharmendra lepromin-induced reduction in ^{3}H-thymidine incorporation in Con-A stimulated peripheral blood mononuclear cell cultures is found in animals resistant to leprosy. In armadillos with disseminated leprosy infections, sonicated Dharmendra lepromin has variable effects depending on the dose of Con-A used to stimulate the cultures.

Clearly there are distinct differences in experimental results using co-culture techniques with *M. leprae* antigens and Con-A to test for suppressor cell phenomena in leprosy patients. These differences have been attributed to the type of *M. leprae* antigen used;[1, 18, 27] the treatment status of the patients;[18] the choice of mitogens, i.e. PHA or Con-A;[27] the final percent serum supplement in the culture medium;[27] and the duration of the *in vitro* cultures.[19] The present observations in armadillos would indicate that the concentration of the Con-A mitogen should be added to this list of variables.

Undoubtedly variations in methodology, reagents and experimental design could explain the different results obtained in different laboratories. The *in vitro* assay system is technically simple and direct, but the biological events occurring in the mononuclear cell cultures concomitantly stimulated with antigen and mitogen are clearly complex. Blastogenic responsiveness to Con-A alone *in vitro* is influenced by a variety of factors[8] and constituents of mycobacteria in general can suppress lymphocyte blastogenesis directly.[28] For example, mycobacterial D-arbino-D-mannan purified from culture filtrates of *M. tuberculosis* can suppress lymphocyte responsiveness. This suppression is not contingent upon prior exposure of individuals to *M. tuberculosis* and the generation of suppressor cells is not implicated.[7] Bjune[2] has observed that some preparations of *M. leprae* (sonicated bacilli) contain factor(s) able to suppress lymphocyte responsiveness *in vitro* in a fairly non-specific way. Furthermore, using co-culture techniques with combinations of PHA and *M. leprae* sonicate, this investigator[3] concluded that the results are ‘a non-specific phenomenon not related to antigen sensitized lymphocytes’. Indeed, it is not out of the question that some of the observations made in this study could be related to a peculiarity of the Con-A system. Like all

lectins, Con-A will bind to sugars in solution. It has been shown that Con-A binds strongly to D-arabinomannan found in culture filtrates of *Mycobacterium tuberculosis*.[5] It is possible that polysaccharides derived from sonicated Dharmendra antigen when added simultaneously with Con-A, absorbed significant amounts of Con-A and reduced its effective stimulatory concentration. This could explain the shift in the Con-A dose response curve as illustrated in Figure 1(a) in resistant armadillos. However, generally 72-hr mononuclear cell cultures from resistant armadillos when first exposed to 3·16 μg of Con-A for 16 hr and then exposed to 15 μg of sonicated Dharmendra antigen, gave a response less than that in 72-hr replicate mononuclear cell cultures exposed for 56 hr to 3·16 μg of Con-A. Furthermore, 72-hr mononuclear cultures from resistant armadillos when first exposed to 15 μg of sonicated Dharmendra antigen for 16 hr and then exposed to 3·16 μg of Con-A, gave a response far less than 72-hr replicate mononuclear cell cultures exposed for 56 hr to 3·16 μg of Con-A (data not shown). Although Con-A was only tested at one concentration, the staggered addition of these two reactants still shows a suppressive effect exerted by sonicated Dharmendra antigen on mononuclear cell cultures from resistant armadillos. In susceptible armadillos, 72-hr mononuclear cell cultures first exposed to 3·16 μg of Con-A for 16 hr and then exposed to 15 μg of sonicated Dharmendra antigen, show an augmented response when compared to 72-hr replicate mononuclear cell cultures exposed for 56 hr to 3·16 μg of Con-A. Seventy-two hour mononuclear cell cultures from susceptible armadillos, when first exposed to 15 μg of sonicated Dharmendra antigen for 16 hr and then exposed to 3·16 μg of Con-A, gave a response similar to 72 hr replicate mononuclear cell cultures exposed for 56 hr to 3·16 μg of Con-A.

In short, such co-culture experiments, while appearing to be quite simple and direct, are probably influenced by a variety of factors and a variety of cells, helper T, suppressor T, effector T, antigen processing by macrophages, suppressor macrophages, suppressor B lymphocytes, etc., each responding with a variety of receptors to potentially different antigens of *M. leprae* and each T subset probably having complex dose–response relationships to Con-A. Thus, considerable reservations are in order regarding whether or not this experimental system reflects *in vivo* phenomena.

Within the limits of this system, the most striking finding in the present study is the consistent depression of responses by sonicated Dharmendra antigen in resistant armadillos at all three concentrations of Con-A. This would imply that suppressor mechanisms are operative in resistant animals under these conditions *in vitro*. We have also observed depression of responses by Dharmendra antigen and sonicated Dharmendra antigen using the same *in vitro* assay system in leprosy patients with potential resistance (tuberculoid cases) and in individuals with presumably effective immunity (lepromin positive normal individuals).[23] To the extent that these results can be extrapolated to the *in vivo* situation, these suppressor cells teleologically probably exist to protect the host against

delayed-type hypersensitivity reactions (Type I or reversal-type reactions in borderline and tuberculoid leprosy clinically).

M. leprae may activate suppressor cells *in vitro* in susceptible armadillos. One could interpret the data obtained at a dose of 0·78 μg/well of Con-A (Figure 1(a), Table 2) in this fashion. If this finding can be extrapolated to the *in vivo* situation, these suppressor cells may operate on protective cell-mediated immunity. A more likely explanation in our view on teleologic grounds, is that these suppressor cells operate to decrease antibody responses to *M. leprae* antigens and may function in preventing humoral immune responses harmful to the host, i.e. Type II or erythema nodosum leprosum reactions occurring in lepromatous leprosy clinically. The enhanced responses seen in leprous armadillos in the *in vitro* co-culture experiments at a high concentration of Con-A are in agreement with the observations of Nath & Singh[18] in human lepromatous leprosy and may represent concomitant stimulation of an expanded clone of B lymphocytes in this system. These B lymphocytes could be reacting to portions of the complex antigenic mosaic of *M. leprae* which may be the same or may be quite different from those involved in protective cell-mediated immune responses.

This technically simple system, in our view, is far too biologically complex to allow differentiation of these and probably any number of equally valid alternate possibilities.

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