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Hydrogen peroxide and superoxide production by peripheral blood monocytes in leprosy

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SUMMARY

Susceptibility to infection with *Mycobacterium leprae*, the causative organism of leprosy, is the result of a defect in cell-mediated immunity (CMI). The co-operation of macrophages and T lymphocytes is known to be essential for competent CMI response. In this study we have examined peripheral blood monocytes from a range of leprosy patients in an attempt to identify a possible defect in macrophage function. The ability of these cells to produce hydrogen peroxide and superoxide, two bactericidal metabolites of the monocyte/macrophage, has been measured. Monocytes from leprosy patients were found to be capable of producing normal amounts of hydrogen peroxide and superoxide, and no differences in production were found between tuberculoid, lepromatous and control monocytes. These results suggest that macrophages in leprosy are competent, and that probably a T lymphocyte defect contributes to susceptibility to this disease.

Keywords hydrogen peroxide superoxide monocytes leprosy

INTRODUCTION

Mycobacterium leprae infection in man results in a spectrum of disease the form of which is thought to depend upon the cell-mediated immune (CMI) response of the host (Turk, 1969). The lepromatous form of leprosy is characterized by a heavy widely disseminated bacterial load, and poor CMI to *M. leprae* antigens, whilst at the other end of the spectrum the comparatively effective CMI of the tuberculoid patient restricts bacillary growth (Ridley & Jopling, 1966).

Competent CMI is known to require the co-operation of both T lymphocytes and macrophages (Mackaness, 1969) and a number of studies have attempted to identify a defect in these cell types in lepromatous leprosy.

Recently it has been shown that gamma interferon, a macrophage activating factor, is produced in lower levels by lepromatous T lymphocytes than by T lymphocytes from tuberculoid patients or controls (Nogueira *et al.*, 1983). It is possible that as a result of this depressed gamma interferon production, lepromatous macrophages are not activated in the manner required to control intracellular growth of *M. leprae*.

Activation of macrophages results in the induction or augmentation of a number of cellular functions including production of hydrogen peroxide (H_2O_2) and superoxide (O_2^-). The production of these potentially bactericidal metabolites has been correlated with the intracellular killing of *M. microti* (Walker & Lowrie, 1981), *Trypanosoma cruzi* (Nathan *et al.*, 1979) and *M. tuberculosis* (Jackett, Aber & Lowrie, 1978): *M. leprae* has been shown to be susceptible to H_2O_2 *in vitro* (Klebanoff & Shepard, 1984; Sharp, Colston & Banerjee, 1985). The purpose of this study was to

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examine macrophages from leprosy patients throughout the spectrum in an attempt to correlate lack of activation of macrophages with depressed H_2O_2 and O_2^- production and the growth of *M. leprae* that is observed in the lepromatous patient.

MATERIALS AND METHODS

Patients. Leprosy patients for this study were selected by Dr G. Ramu and colleagues from those attending the Out Patients Department at the Central JALMA Institute for Leprosy, Agra, India. Patients were included in the study after clinical classification according to Ridley & Jopling (1966): the majority had received no treatment. All but two patients were male, and their ages ranged from 16 to 70 years. Healthy controls were laboratory workers from JALMA.

Preparation of monocyte cultures. Mononuclear leucocytes were isolated from heparinized venous blood using Ficoll-Hypaque as previously described (Wynne & Moore, 1982). Cell suspensions were dispensed into 96 well microtitre trays (Sterilin); after a 2–3 h period of incubation at 37°C in 5% CO_2 in air, they were washed thoroughly in Hanks' balanced salt solution (HBSS) without phenol red, after which adherent cells were considered to be monocytes.

Assays for H_2O_2 and O_2^- release. H_2O_2 and O_2^- assays were carried out as previously described (Pick & Keirsari, 1980; Pick & Mizel, 1981). For the H_2O_2 assay, medium was replaced by 0·1 ml reaction mixture containing phenol red (0·1 g/l) and 19 u/ml horseradish peroxidase (Type II) in HBSS. Ten microlitres of 1 M NaoH was added to a control well at time zero to stop the reaction and give a baseline reading. The plates were incubated at 37°C in 5% CO_2 in air for 90 min after which 10 μl of 1M NaoH was added to a second well containing the reaction mixture. Phorbol myristate acetate (PMA) was added to a third well at a final concentration of 1 μg/ml, in reaction mixture. After 90 min incubation, the reaction was stopped by the addition of 10 μl of 1M NaoH. *M. leprae* at 1×10^8/well in reaction mixture was added to two further wells. Ten microlitres of 1 M NaoH was used to stop the reaction in the first of these at time zero and in the second, after 90 min incubation. Changes in absorbance were read at 600 nm and a standard curve was prepared from which results in terms of nanomoles of H_2O_2 could be established. An automatic immuno assay reader (Multiscan—generously loaned for the duration of this study by the manufacturers, Flow Laboratories, Irvine, UK) was used to read absorbance changes.

Macrophages were cultured and washed in the same manner for assay of O_2^- production. They were incubated for 90 min in the presence of 0·1 ml 160 μM ferricytochrome C (Type III) in HBSS, alone, or supplemented with either 300 u/ml superoxide dismutase (baseline reading) or with PMA as above, or with *M. leprae* with and without superoxide dismutase. Changes in absorbance were measured at 500 nm. The amount of O_2^- per well is expressed by the formula:

$$\text{nmol } O_2^- = \frac{\text{absorbance at 550 nm} \times 100}{6{\cdot}3}.$$

All reagents were supplied by Sigma.

Results were compared by the Kruskal–Wallis one way analysis of variance by ranks (Siegal, 1956).

RESULTS

In this study, peripheral blood monocytes from patients throughout the leprosy spectrum, and healthy controls were examined for their ability to produce H_2O_2 or O_2^- in the resting state, or after stimulation with the membrane perturbant PMA or after phagocytosis of *M. leprae*. Table 1 shows H_2O_2 production by these cells. It can be seen that resting cells produce very little H_2O_2 but that after PMA stimulation, monocytes from all groups produced comparatively large amounts of H_2O_2. However, no differences could be detected in the ability of macrophages throughout the leprosy spectrum to produce H_2O_2 ($P = 0{\cdot}8$). Similarly stimulation of monocytes with *M. leprae* resulted in raised H_2O_2 production when compared to resting cells, though this increase was not as great as with PMA. Again, no significant difference in H_2O_2 production was detected in monocytes isolated

Table 1. H_2O_2 production by peripheral blood monocytes in leprosy

Type of leprosy*	H_2O_2 production (nmol/5 × 10^5 cells) in reponse to					
	Resting†		*M. leprae*†		PMA†	
LL	0·1		1·1		2·1	
LL	0·6		1·2		5·3	
LL	0·2		0·7		5·4	
LL	0	$\bar{x}=0·3$	2·9	$\bar{x}=1·2$	11·3	$\bar{x}=4·1$
LL	0		0·1		1·2	
LL	0·6		1·5		4·7	
LL	1·1		0·8		2·1	
LL	0		0		0·9	
BL	0·1	$\bar{x}=0·1$	1·3	$\bar{x}=1·3$	2·1	$\bar{x}=2·1$
BB	0·1		0		7·2	
BB	1·7	$\bar{x}=0·6$	4·4	$\bar{x}=2·3$	3·4	$\bar{x}=4·8$
BB	0·2		2·4		3·7	
BT	1·1		3·4		18·0	
BT	0		0·7		3·7	
BT	0	$\bar{x}=0·4$	0·4	$\bar{x}=1·2$	1·4	$\bar{x}=5·8$
BT	1·1		2·2		7·7	
BT	0		0		0·7	
BT	0·2		0		3·9	
Control	1·0		0		6·1	
Control	0	$\bar{x}=0·4$	1·7	$x=0·5$	1·9	$\bar{x}=3·2$
Control	0·2		0		2·6	
Control	0·3		0·4		2·3	

* Based on Ridley–Jopling classification: LL = lepromatous leprosy; BL = borderline lepromatous leprosy; BB = borderline leprosy; BT = borderline tuberculoid leprosy.

† $P>0·1$

from patients having polar or intermediate forms of leprosy, or healthy controls ($P=0·6$). A similar pattern of response was observed when O_2^- production by monocytes derived from a different group of leprosy patients was measured (results not shown). As with H_2O_2, resting cells produced very little O_2^- but production was greatly increased in all groups after stimulation with PMA and to a lesser extent, with *M. leprae*. Again, no differences were observed between groups within the leprosy spectrum or the control group ($P=0·3$ with PMA, $P=0·8$ with *M. leprae*, $P=0·1$ with resting cells).

DISCUSSION

Activated macrophages are known to be important in the control of intracellular infections (Birmingham & Jeska, 1981; Mackaness, 1969; Nathan *et al.*, 1979) and it is thus possible that a macrophage defect could account for the breakdown in CMI which is observed in lepromatous leprosy. Unresponsiveness of macrophages to activation or failure to become sufficiently activated could result in the extensive intracellular growth of *M. leprae* that is a characteristic of lepromatous leprosy. Elevated production of H_2O_2 and O_2^- are parameters of macrophage activation and H_2O_2 production has been correlated with the intracellular killing of two mycobacterial species (Jackett *et al.*, 1978; Walker & Lowrie, 1981). In addition, *M. leprae* has been shown to be susceptible to the bactericidal effects of H_2O_2. Thus it seemed possible that in tuberculoid macrophages increased O_2^- and H_2O_2 production might be reflected by the ability to control (at least to some extent) growth of *M. leprae*: in contrast, in macrophages from lepromatous patients *M. leprae* might multiply in an environment depleted of bactericidal activity.

Our findings show that this is not the case since although monocytes from all patients studied produced very little H_2O_2 or O_2^- in the resting state, production of these two bactericidal metabolites was significantly increased after stimulation with PMA or *M. leprae*. Thus monocytes from lepromatous patients are potentially capable of activation at least as far as H_2O_2 and O_2^- production are concerned, and thus have the theoretical ability to kill *M. leprae*.

The production of H_2O_2 and O_2^- by peripheral blood monocytes could not be correlated with the position of the cell donor in the leprosy spectrum: statistical analysis showed that there was no significant difference between groups in the Ridley and Jopling scale. These findings are in accordance with a recent study (Horwitz, Levis, & Cohn, 1984) in which tuberculoid and lepromatous monocytes were compared in terms of their ability to kill the intracellular bacterial pathogen *Legionella pneumophila*. Monocytes from both groups were found to inhibit intracellular multiplication equally well. These monocytes, in contrast to those in the present study, had been activated by pre-incubation with concanavalin A stimulated T cell supernatants. Thus it would appear that monocytes in leprosy are receptive to activation by T cell products and have the potential to produce bactericidal metabolites and prevent multiplication of intracellular organisms. In these respects, it would seem that the differences in CMI which commit a patient to one or other form of leprosy are to be found in the T cell compartment of CMI, as has been indicated (Nogueira *et al.*, 1983) and that monocytes in leprosy are normal in terms of their bactericidal capacity, but are not being properly activated to kill *M. leprae*.

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