Indian J Chest Dis & All Sci
Vol. 31 No. 3 pp 141-149 (1989)

HYDROGEN PEROXIDE RELEASE BY OKIa1 (ANTI DR--MONOCLONAL ANTIBODY) RESISTANT ALVEOLAR MACROPHAGES IN TUBERCULOSIS

P. Selvaraj, Rajiswamy, V.K. Vijayan, R. Prabhakar and P.R Narayanan

Tuberculosis Research Centre, Madras

Phorbol myristate acetate (PMA) triggered hydrogen peroxide (H_2O_2) release from alveolar macrophages and corresponding blood monocytes were studied as a whole, in active tuberculosis, inactive tuberculosis (treated), non-tuberculous lung disease patients and normal individuals. Irrespective of the study subjects, the alveolar macrophages produced less H_2O_2 than the corresponding blood monocytes. The alveolar macrophages that were resistant to OKIa1 (Anti-DR monoclonal antibody and complement treatment) produced an increased level of H_2O_2 than the control ascites and complement treated alveolar macrophages. Moreover, such increase in H_2O_2 release was not seen with peripheral blood monocytes; more than 90% monocytes were OKIa1 resistant population. These OKIa1 resistant alveolar macrophages are probably important in their metabolic, microbicidal and the immunological functions.

Key Words : H_2O_2 release, human alveolar macrophages, blood monocytes, OKIa1 resistant alveolar macrophages.

Alveolar macrophages are the mononuclear phagocyte population lining the alveoli of the lung. These cells are mainly involved in killing and eliminating the pathogens.[1] One of the killing mechanisms is the respiratory burst. The capacity of these cells to produce reactive oxygen intermediates upon stimulation (respiratory burst) is probably related to the cellular capacity for microbicidal function; which represents one of the host defense function against intracellular pathogens[2-6]. The reactive oxygen intermediates such as superoxide anions and hydrogen peroxide are well known for their highly toxic oxidant activity for pathogens. It has been shown that macrophages and monocytes express membrane receptors for phorbol myristate acetate (PMA)[7,8]. When these cells are

Correspondence: Dr P.R. Narayanan, Deputy Director (Immunology), Tuberculosis Research Centre, Spur Tank Road, Chetput, Madras-600 031.

stimulated with PMA, the YMA triggers the cells to produce superoxide anions and H_2O_2 through NADPH oxidase system.

It has been shown that alveolar macrophages are less efficient in their immune functions such as antigen presentation to T-lymphocytes and interleukin-1 production[9-11]. It has been mentioned that the alveolar cells are immunologically inert[12].

It is well established that the immune region associated Ia antigens are responsible for the immunological functions such as antigen presentation[13] and mixed leucocyte reaction[14]. Based on the phagocytic function, macrophage heterogeneity has been shown among the Ia^+ alveolar macrophages in disease status[15]. On the other hand, in a study carried out in the blood monocytes of tuberculosis patients, an increased level of Ia negative monocytes have been shown. The same study also suggested that HLA-DR negative and -DR positive phenotypes represent different stages of maturation[16].

In our earlier report we have shown that the PMA induced H_2O_2 production by the alveolar macrophages was similar among the active tuberculosis, inactive tuberculosis and non-tuberculous lung disease patients. Further, the alveolar macrophages and the blood monocytes of the active pulmonary tuberculosis patients and those of normal controls were equally competent in producing H_2O_2, *in vitro,* upon stimulation with PMA[17].

In the present study, the PMA triggered H_2O_2 producing potential of alveolar macrophages and the corresponding blood monocytes were compared. Further, the H_2O_2 producing potential of an alveolar macrophage population, which was resistant to OKIa1 (anti-DR monoclonal antibody) monoclonal antibody was studied. Even though the study was carried out as a whole, the finding may be useful to understand the role of OKIa1 resistant alveolar macrophages in their microbicidal and immunological functions to the intracellular pathogens such as *M. tuberculosis.*

Material and Methods

Study Subjects. The patients were suspected cases of pulmonary tuberculosis attending Tuberculosis Research Centre with respiratory symptoms and radiographic evidence of lung diseases of varying duration. All the patients had at least six sputum smears negative for *M. tuberculosis* by microscopy. All patients sputa were cultured for *M. tuberculosis* and depending upon the culture results, these patients were classified into 3 groups.

Group 1. Active pulmonary tuberculosis. These patients had not received any previous anti-tuberculosis chemotherapy and had grown *M. tuberculosis in* one or more sputum specimens in culture (6 patients).

Group 2. Inactive pulmonary tuberculosis. These patients had had previous anti-tuberculosis chemotherapy and presently their sputum showed culture negativity for *M. tuberculosis* (5 patients).

Group 3. Non-tuberculous lung disease. This group involved patients with lung abscesses, bacterial pneumonia and pulmonary manifestations of *polycythemia vera* (2 patients).

Group 4. Normal subjects who had normal chest x-ray and pulmonary functions and were non-smokers (2 individuals).

Fibreoptic Bronchoscopy and Alveolar Lavage. Fibreoptic bronchoscopy procedure[18] was performed in the affected lobe and two other lobes, usually middle lobe and lingula. Each lobe was lavaged with 100 ml isotonic sterile saline and the lavage samples were pooled and the mucus was eliminated by filtration.

Alveolar Macrophages and Peripheral Blood Mononuclear Cells. The alveolar macrophages of the lavage fluids were used for the hydrogen peroxide assay. The percentage dead cells were from 10-15% as judged by trypan blue dye exclusion method. The per cent neutrophils and eosinophils were, <3% and <2%, respectively. The alveolar cells were washed in RPMI-1640 culture medium and resuspended in the same medium.

Peripheral blood mononuclear cells were separated using Ficoll-Hypaque as described elsewhere[19]. The cells were washed thrice in RPMI medium and adjusted to 5 x 10^6 cells/ml medium.

Non-Specific Esterase Staining. Smears of peripheral blood mononuclear cells were stained for non-specific esterase for determining the percentage of monocyte population according to the method of Horwitz[20]. A total number of 300 cells were counted and the per cent monocytes (esterase positive cells) among them were determined.

Cytotoxicity Assay. HLA-DR negative alveolar macrophages were selected using antibody and complement mediated conventional cytotoxicity assay by the method described by Whisler, *et al* [21]. Briefly, alveolar cells were incubated for 1 hr, at 4°C with 1 : 100 final dilution of OKIa1 antibody in RPMI medium with 5% Foetal calf serum (Ortho Diagnostic Systems Inc., U.S.A.) (an anti-HLA-DR antigen frame work complex expressed on B-cells, activated T-cells and monocytes). Then the cell suspension was rinsed in RPMI containing 5% foetal calf serum and was further incubated for 1 hr at 37°C with 1: 4 final dilution of rabbit complement. The cells were washed twice in RPMI medium. Cells treated with control ascites and complement served as control. The percentage of cells susceptible to OKIa1 monoclonal antibody and complement treatment was assessed by trypan blue dye exclusion method and expressed as per cent cytotoxicity. Blood monocytes were also treated in the same way. The alveolar macrophages and the blood monocytes which were resistant to OKIa1 and complement treatment (HLA-DR negative cells) and the corresponding control macrophages were studied for their hydrogen peroxide producing potential. More than 95% of the OKIa1 resistant alveolar cells were macrophages, as judged by Diff-quick staining and were all non-specific esterase positive.

H_2O_2 Assay. Hundred microliter containing 0.1 x 10^6 alveolar macrophages, or OKIa1 resistant alveolar macrophages/monocytes or, the control alveolar macrophages/

monocytes were plated in 96 well plate. For peripheral blood, 0.5 x 10^6 blood mononuclear cells were seeded in 96 well flat bottom plates (Falcon) and non-adherent cells were removed by aspiration after one hour at 37°C. The adherent mononuclear cells (non-specific esterase positive cells) and the alveolar macrophages were used for H_2O_2 assay.

The H_2O_2 assay method described by Pick and Mizel[22] was followed. Briefly, the plate with the cells were washed with phenol red free Hank's balanced salt solution (HBSS). 100 µl of HBSS containing 19 units/ml horse radish peroxidase (Type-III, Sigma), 1 µg/ml phorbol myristate acetate (PMA) (Sigma) and 0.02% phenol red (Sigma) were added to each well. After incubating the plate at 37°C for 1 hr the enzyme reaction was arrested with 10 µl of 1M NaOH. Absorbance at 605 mu was determined spectrophotometrically for the assay mixture pooled from live wells. For each experiment a standard was set up in the range of 0-10 n mol/ml of H_2O_2.

Results

In the present study, the alveolar macrophages of active tuberculosis, inactive tuberculosis, non-tuberculous lung disease patients and normal subjects produced less phorbol myristate acetate induced hydrogen peroxide when compared to the corresponding blood monocytes (Table 1).

The alveolar macrophages that were susceptible to OKIa1 monoclonal antibody and complement varied from 15-86%. The per cent susceptibility is expressed as per cent cytotoxicity. The alveolar macrophage population which was resistant to OKIa1

Table 1. *PMA induced hydrogen peroxide production by the alveolar macrophages and blood monocytes in various groups of patients and normal individuals*

		Alveolar macrophages[@]	Blood monocytes[@]
Active TB	1)	2.0	8.2
	2)*	6.2	10.1
	3)*	12.3	5.1
	4)	3.9	7.9
	5)*	3.8	4.7
	6)	2.0	4.9
Inactive TB (Treated)	7)	4.0	12.0
	8)	2.9	4.5
Non-TB lung disease	9)	10.8	27.1
	10)	2.6	10.3
Normal	11)	1.3	18.2

N.B. : [@]PMA induced hydrogen peroxide production in nmols per 0.1x10^6 alveolar macrophages or blood monocytes.
Smokers

monoclonal antibody and complement treatment produced more hydrogen peroxide than the control ascites and complement treated population and the cells which were not subjected to any treatment (Table 2). On the other hand, peripheral blood monocytes which were resistant to OKIa1 monoclonal antibody produced an equal or slightly increased/decreased level of hydrogen peroxide upon stimulation with PMA (Table 3). More than 90% of the blood monocytes were resistant to OKIa1 monoclonal antibody and complement treatment.

Discussion

In the present study, the alveolar macrophages produced less H_2O_2 than the blood monocytes (Table 1). This lesser production of H_2O_2 could be due to the maturation of monocyte/macrophage lineage which ultimately become less efficient. Majority of the alveolar macrophages are felt to be derived from circulating blood monocytes[23,24]. A large body of evidences showing that alveolar macrophages are less efficient in terms of antigen presentation[9], interleukin-1 production[10,11], than the blood monocytes. At the same time, when the monocytes mature *in vitro,* they lose the ability to elaborate IL-1[11]. Whereas, surface expression of HLA-DR class II MHC determinant was increased[25] or, decreased[26]. It has been shown that 6 days old monocyte derived macrophages and pulmonary macrophages failed to function as accessory cells, which suggests that monocytes, as they mature, become less efficient in their functions[12].

Nakashima, *et al* [8] have shown that the PMA and the Con-A triggered, receptor mediated, superoxide release was higher in the blood monocytes than the alveolar macrophages. Further, it has been shown that blood monocytes, as they are in culture for more than 3 days, the H_2O_2 production from day 4 onwards started decreasing which suggests the maturation of monocytes to macrophages[27].

The alveolar macrophages which were resistant of OKIa1 monoclonal antibody and complement treatment produced an increased H_2O_2 than control ascites and complement treated population. This resistant population of macrophages may be either a subset of macrophages which are not susceptible to OKIa1 antibody and complement treatment or may be immature in nature not expressing the -DR antigens recognizable by OKIa1 antibody. Albrechtsen[28] studied human monocytes by using a cytotoxicity assay and an anti HLA-DR hetero-antiserum and concluded that there was a sub-population of such cells not lysed under the conditions of the assay, representing either Ia-negative monocytes or cells with a quantity of surface Ia beneath the sensitivity. In a study carried out in blood monocytes of tuberculosis patients and normal healthy individuals, it has been suggested that HLA-DR negative and DR-positive phenotypes represent different stages of maturation. These DR-negative monocytes were also found to reduce the lymphocyte response to purified protein derivative (PPD) of *M. tuberculosis,* both in tuberculosis patients and healthy subject[16].

Moore and co-workers[25] found that the density of HLA-DR surface expression was high on alveolar macrophages than the monocytes. It has been substantiated that monocytes

Table 2. *PMA induced hydrogen peroxide producing potential of OKIa1 resistant alveolar macrophages*

		H_2O_2 in nmols/0.1 x 10^6 alveolar MØs			
		% Cytotoxicity[@]	Alveolar cells[$]	Control ascites + complement[$]	OKIa1 antibody + complement[$]
Active TB	*1)	86.1	2.4	2.7	7.0
	*2)	44.3	3.8	3.9	5.3
Inactive TB	*3)	42.1	2.0	3.5	9.0
(Treated)	4)	52.5	3.5	5.0	15.0
	*5)	37.5	4.0	8.0	13.6
	6)	45.4	4.4	6.7	7.9
	7)	15.0	3.9	6.3	10.6
Non-TB lung	*8)	58.0		6.8	8.7
diseases	*9)	62.9	2.1	4.3	17.0
Normals	10)	60.0	1.3	1.3	2.2
	11)	47.0	2.8	43	8.7

N.B. : [@] Per cent alveolar macrophages susceptible to OKIa1 monoclonal antibody and rabbit complement treatment.

[$] Hydrogen peroxide production in nmols per 0.1 x 10^6 alveolar macrophages, either the whole cells or mouse ascites and complement treated or, OKIa1 and complement treated cells.

l Smokers.

Table 3. *PMA induced H_2O_2 release by OKIa1 resistant peripheral blood monocytes*

	H_2O_2 in nmols/0.1x 10^6 blood monocytes	
	Control ascites + complement	[@]OKIa1 antibody + complement
Normal	18.5	19.8
Active TB	8.2	7.0
Non-TB	10.3	7.8
Non-TB*	7.0	7.5

N.B. : [@] Per cent peripheral blood monocytes susceptible to OKIa1 monoclonal antibody and complement were 6-10%.

l Smoker, Other are non-smokers.

cultured more than 8 days express Ia-antigens and such cells are equivalent to that of pulmonary macrophages in terms of their biochemical and functional aspects such as antigen presentation to T-cells[12].

In the present study, more than 90% of the peripheral blood monocytes were resistant to OKIa1 monoclonal antibody. The increased production of H_2O_2 by the blood monocytes than the alveolar macrophages could probably be due to their high level (>90%) of OKIa1 resistant population of monocytes in the blood.

The alveolar macrophages which were resistant to OKIa1 may be a newly recruited monocyte-macrophage lineage not expressing a detectable level of Ia antigens on their surface. It will be useful to study the role of these cells in their microbicidal and immunological functions against intracellular pathogens.

Acknowledgement

The authors sincerely thank Mrs Annamma Joy and Mr K. Sankaran for their help in the procedure of FFB and BAL; and Mr N. Venkataprasad for his help during this study and Mr P. Karthigayan for typing the manuscript.

References

1. Green GM, Kass EH. The role of the alveolar macrophage in the clearance of bacteria from the lung. *J Exp Med* 1964; **119** : 167-175.
2. Jackett PS, Aber VR, Lowrie DB. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *M. tuberculosis. J Gen Microbiol* 1978; **104** : 3745.
3. Nathan C, Nogueira N, Juangbhanich C, Ellis J, Cohn Z. Activation of macrophages *in vivo* and *in vitro*. Correlation between hydrogen peroxide release and killing of Tryupanosoma cruzi. *J Exp Med* 1979; **149** : 1056-1068.
4. Walker L, Lowrie DB. Killing of *M. microti* by immunologically acitivated macrophages. *Nature* 1981; **293** : 6970.
5. Klebanoff SJ, Shepard CC. Toxic effect of the peroxidase-hydrogen peroxide halide anti-microbial system on *Mycobacterium leprae. Infect Immun* 1984; **44** : 534-536.
6. Murray HW, Rubin BX, Carriero SM, Harris AM, Jaffee EA. Human mononuclear phagocyte antiprotozoal mechanisms : Oxygen-dependent vs Oxygen-independent activity against intracellular *toxoplasma gondii. J Immunol* 1985; **134:** 1982-1988.
7. Hoidal JR, Repine JE, Beall GD, Rasp FL, White JG. The effect of phorbo1 myristate acetate on the metabolism and ultrastructure of human alveolar macrophages. *Am J Pathol* 1978; **91:** 469-482.
8. Nakashima H, Ando M, Sugimoto M, Suga M, Soda K, Araki S. Receptor mediated O_2 release by alveolar macrophages and peripheral blood monocytes from smokers and non-smokers. Priming and triggering effects of monomeric IgG, Concanavalin-A, N-formyl-Methionyl-Leucyl-Phenylalanine, Phorbol myristate acetate and cytochalasin-D. *Am Rev Respir Dis* 1987; **136** : 310-315.

9. Ettensohn DB, Robbert J (Jr). Human alveolar macrophage support of lymphocyte response to mitogens and antigens : Analysis and comparison with autologous peripheral blood derived monocytes and macrophages. *Am Rev RespirDis* 1983 ; **128** : 516-522.

10. Wewers MD, Rennard SI, Hance AI, Bitterman PB, Crystal RG. Normal human alveolar macrophages obtained by bronchoalveolar lavage have a limited capacity to release interleukin-1. *J Clin Invest* 1984; **74** *:* 2208-2218.

11. Elias JA, Schreiber AD, Gustilo K, *et al*. Differential interleukin-1 elaboration by unfractionated and density fractionated human alveolar macrophages and blood monocytes: Relationship to cell maturity. *J Immuno* 1985; **135** : 3198-3204.

12. Mayemik DG, Ul-Haq A, Rinehart JJ. Differentiation-associated alteration in human monocyte - macrophage accessory cell function. *J Immunol* 1983; **130** : 2156-2160.

13. Benacerraf B. Role of MHC gene products in immune regulation. Science 1981; **212** : 1229-1238.

14. Rossi GA, Zocchi E, Sacco O, Balbi B, Ravazzoni C, Damiani G. Alveolar macrophage stimulation of T-cell proliferation in autologous mixed lymphocyte reactions. Role of HLA-DR antigens. *Am Rev Respir Dis* 1986, **133** : 78-82.

15. Gant VA, Hamblin AS. Human bronchoalveolar macrophage heterogeneity demonstrated by histochemistry, surface markers and phagocytosis. *Clin Exp Immunol* 1985; **60** *:* 539-545.

16. Tweardy DJ, Schaaer BZ, Ellner JJ. Association of alter dynamics of monocyte surface expression of human leucocyte antigen DR with immunosuppression in tuberculosis. *J Infec Dis* 1984; **149** : 3137.

17. Selvaraj P; Rajiswamy, Vijayan VK, Prabhakar R, Narayanan PR. Hydrogen peroxide producing potential of alveolar macrophages and blood monocytes in pulmonary tuberculosis. *Ind J Med Res* 1988, **88** *:* 124-129.

18. Hunninghake GW, Gadek JE, Kawanami O, Ferrans VJ, Crystal RJ. Inflammatory and immune processes in the human lung in health and disease : evaluation by bronchoalveolar lavage. *Am J Path01 1979,97 :149-2C6.*

19. Boyum A. Separation of leukocytes from blood and bone marrow. *Stand J Clin Lab Invest* (Suppl) 1968; 21 (Suppl97) : 97-102.

20. Howitz Da, Allison AC, Ward P, Knight N. Identification of human mononuclear leucocyte populations by e&erase staining. Clin Exp *Immunoll9n, 30 : 289-298.*

21. Whisler RL, Newhouse YG, La&man LB. Differential abilities of human peripheral blood monocytes quantitatively or qualitatively differing in HLA-DR and HLA-DS expression to support B-cell activation in liquid and semisolid cultures. *J Immunol1985,l35 :* 172179.

22. Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by cells in culture using an automatic enzyme immunossary reader. *J hnmunol Methods* 1981; 46 : 211226.

23. Blusse Van Oud Albas AB, Van Der Linden - Schrever, Van Furth R. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intracellular administration of aerosolized heat killed BCG. *Am Rev Respir Dis* 1983; **128** : 276-281.

24. Bitterman PB, Saltzman LR, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication : One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. *J Clin Invest* 1984, **74** : 460-469.

25. Moore ME, Rossman MD, Schreiber. AD, Douglas SD. DR frame work antigens and 63D3 antigens on human peripheral blood monocytes and alveolar macrophages. *Clin Immunol Immunophathol* 1983; **29:** 119-128.

26. Razma AG, Lynch JP III, Wilson BS, Ward PA, Sunkel SL. Expression of Ia, like (DR) antigen on human alveolar macrophages isolated by bronchoalveolar lavage. *Am Rev Respir Dis* 1984, **129:** 419-424.

27. Nakagawara A, Nathan CF, Cohn ZA. Hydrogen peroxide metabolism in human monocytes during differentiation *in vitro. J Clin Invest* 1981; **68** : 1243-1252.

28. Albrechtsen D. HLA-D associated Ia like antigens on human macrophages. *Scand J Immunol* 1977; **6** : 907-912.

For Reprints : Dr R. Prabhakar, Director, Tuberculosis Research Centre, Spurtank Road, Chetput, Madras600 031.