Immunology 1989 **66** 451–458

Generation of superoxide anion by alveolar macrophages in sarcoidosis: evidence for the activation of the oxygen metabolism in patients with high-intensity alveolitis

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Accepted for publication 3 October 1988

SUMMARY

We studied superoxide anion (O_2^-) generation by alveolar macrophages (AM) isolated from bronchoalveolar lavages (BAL) of patients with sarcoidosis, and assayed immediately after the isolation or after maintenance in culture for 2 days. In assays of cells freshly isolated from BAL, AM of patients with active sarcoidosis with a high-intensity lymphocytic alveolitis produced more O_2^- in response to phorbol myristate actetate than AM of patients with inactive sarcoidosis. Also, after 2 days of cultivation sarcoid AM were heterogeneous in their capability to metabolize oxygen, although both AM of active and inactive sarcoid patients produced higher amounts of O_2^- than AM of healthy subjects. *In vitro* treatment with recombinant interferon-gamma (rIFN-γ) caused an enhancement of the capability of AM of inactive sarcoid patients to produce O_2^- in response to PMA. AM of patients with active sarcoidosis did not respond to rIFN-γ when they already produced O_2^- vigorously. However, they became sensitive to the activating effect of rIFN-γ after the down-modulation of their capability to produce O_2^-, that occurred upon prolonged cultivation. Monocytes isolated from blood of sarcoid patients and assayed immediately or after different times of cultivation did not produce more O_2^- than control monocytes and monocyte-derived macrophages, thus indicating that the activation of AM in sarcoidosis is likely a local phenomenon. These studies strengthen the notion that T lymphocyte–macrophage interaction is a critical event in the pathogenesis of sarcoidosis and establish that the enhanced capability to metabolize oxygen to highly reactive intermediates by AM is one of the consequence of this interaction.

INTRODUCTION

The role of macrophages as effector cells in host defences against invading micro-organisms and tumour cells, and in tissue damage at sites of inflammation, depends to a large extent on the capability to metabolize oxygen to highly reactive intermediates such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH).

Studies performed by using animal models have provided extensive evidence that the capability to metabolize oxygen by tissue macrophages is finely modulated (Rossi, Zabucchi & Romeo, 1975; Nathan, 1985; Berton & Gordon, 1985). Injection into the peritoneal cavity of mice of intracellular pathogens or bacterial-derived products activates the macrophage capability to reduce the oxygen molecule to highly reactive intermediates in response to environmental stimuli.

Human models of activation of macrophage oxygen metabolism are less known. Modulation of the capability of human monocytes and monocyte-derived macrophages to produce O_2^- and H_2O_2 has been studied by *in vitro* exposure to lymphocyte-derived products (Nagakawara *et al.*, 1982) or endotoxin (Pabst, Hedegard & Johnston, 1982). More recently, interferon-gamma (IFN-γ) has been recognized as the T-cell-derived molecule able to activate the capability of human macrophages to metabolize oxygen (Nathan *et al.*, 1983).

One clinically relevant situation where macrophages together with T lymphocytes predominate as cells present at sites of inflammation with chronic evolution is sarcoidosis. In this disease, the most relevant phenomenon is the accumulation of macrophages and T lymphocytes in the lung alveoli with a concomitant inflammatory injury of the alveolus wall (Hunninghake *et al.*, 1984; Semenzato *et al.*, 1986).

Abbreviations: AM, alveolar macrophages; BAL, broncho-alveolar lavage; H_2O_2, hydrogen peroxide; KRP, Krebs Ringer phosphate buffer; KRPCaG, KRP plus 0·5 mM $CaCl_2$ and 5 mM glucose; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; rINF-γ: human recombinant interferon-gamma.

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Since molecules derived from the reduction of oxygen are well known as mediators of lung injury (reviewed by Fantone & Ward, 1984), the activation of the macrophage capability to produce toxic oxygen molecules might play a crucial role in developing the tissue damage in sarcoid lung.

In this paper we present results of studies on the oxygen metabolism of macrophages recovered from the bronchoalveolar lavage (BAL) of patients with sarcoidosis. We show that alveolar macrophages (AM) from sarcoid patients produce higher amounts of superoxide anion upon triggering and that there is a good correlation between capability to metabolize oxygen and the clinical state of the disease. We also provide evidence that IFN-γ could be responsible for the activation of AM oxygen metabolism, thus suggesting that lymphocyte–macrophage interaction and generation of toxic oxygen molecules can play a central role in the pathogenesis of lung damage in this disease.

MATERIALS AND METHODS

Study population

A total of 43 patients (23 men, 20 women; mean age $36{\cdot}6 \pm 11$) were studied. In all cases biopsy material obtained from lung, lymph nodes or livers contained non-caseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous diseases. On the basis of percentage and absolute numbers/ml of T lymphocytes recovered from the BAL and, when available, the positivity of ^{67}Ga scan, the following groups of patients were defined: (i) 23 patients with active sarcoidosis (high-intensity lymphocytic alveolitis: $>28\%$, $>50 \times 10^3$/ml lymphocytes in the BAL) and ^{67}Ga scan positivity; (ii) 20 patients with inactive sarcoidosis (low-intensity lymphocytic alveolitis: $<12\%$, $<25 \times 10^3$/ml lymphocytes in the BAL) and ^{67}Ga scan negativity. All patients with active sarcoidosis, and eight patients with inactive disease, were studied at the time of diagnosis, before any therapy. Twelve patients with inactive sarcoidosis previously received corticosteroids but, at the time of this study, they were off therapy for at least 3 months. All the patients were non-smokers or had given up smoking at least 2 years before the diagnosis. For comparison, four non-smoker healthy volunteers (three men, one woman; mean age 40 ± 7) were studied. The characteristics of the BAL of these subjects are reported in Tables 1 and 2. Differential cell counts and quantification of CD4- and CD8-positive lymphocytes were performed as described by Semenzato *et al.* (1986).

Isolation and cultivation of alveolar macrophages

BAL mononuclear cells obtained as previously described in detail by Semenzato *et al.* (1985) were centrifuged and the pellet resuspended in RPMI-1640 medium (Flow Laboratories, Irvine, Ayrshire, U.K.) containing 50 U/ml penicillin and 50 μg/ml streptomycin. After depletion of T lymphocytes using a rosette method with sheep red cells (Semenzato *et al.*, 1986), AM were washed once in phosphate-buffered saline (PBS). The final pellet was suspended in Krebs Ringer phosphate buffer (KRP; Berton *et al.*, 1985) containing 0·5 mM $CaCl_2$ and 5 mM D-glucose (KRPCaG) when the cells were assayed immediately for O_2^- production or in RPMI-1640 containing 10% fetal calf serum, inactivated at 56° for 30 min, and antibiotics when the cells were cultivated. For cultivation, AM were plated at a density of $2{\cdot}0 \times 10^5$ cells/well in a volume of 0·5 ml in 24-well trays (Space Saver, Flow Labs). The medium was aspirated and replaced with fresh medium every 48 hr. AM adhered firmly to tissue culture plastic and the detachement from the suface of adhesion was negligible also after prolonged cultivation. After 2 days of cultivation the micrograms of adherent proteins per well were: $37{\cdot}1 \pm 9{\cdot}5$ for normal subjects, $54{\cdot}3 \pm 16{\cdot}6$ for inactive sarcoidosis patients, $93{\cdot}0 \pm 33{\cdot}0$ for active sarcoidosis patients. As judged by morphologic observations, these differences were not due to heterogeneity of detachement or survival in culture.

Isolation and cultivation of blood monocytes

Mononuclear peripheral blood cells were isolated by centrifugation of anti-coagulated blood over Ficoll–Paque (Pharmacia, Uppsala, Sweden) by standard procedures. The mononuclear band was then washed in PBS containing 2% pooled human serum and 1·5 mM ethylenediaminetetraacetic acid (EDTA) as described elsewhere (Cassatella *et al.*, 1985) and used for assaying O_2^- production or for cultivation of monocytes as described by Cassatella *et al.* (1985).

Treatment of alveolar macrophages with recombinant interferon-gamma

Recombinant interferon-gamma (rINF-γ; Lot n.H9039A), produced by Genentech Inc., was kindly provided by Boehringer Ingelheim, Wien, Austria. Stock solutions were diluted in RPMI-1640 medium containing 10% fetal bovine serum. The starting solution was declared to contain less than 0·02 EU/ml endotoxin (LPS) and finally diluted in the wells where AM were cultivated about 10^6-fold. Controls done with human monocyte-derived macrophages showed that the activation of the oxygen metabolism obtained by these preparations of rINF-γ was not inhibited by doses of polymyxin B sulphate (1 mg/ml) (Sigma, Taufkirchen, FRG) which blocked the activating effect of 50 ng/ml LPS (*E. coli* type 026:B6 TCA extract, Sigma).

Biochemical assays

O_2^- production was assayed as reduction of ferricytochrome c (Sigma) as originally described by Babior, Kipnes & Curnutte (1973). The method detailed by Berton *et al.* (1985) was used for alveolar and monocyte-derived macrophage monolayers. AM freshly isolated from BAL were assayed as described by Berton & Gordon (1983), in 24-well trays: 2×10^5 cells in 0·05 ml of KRPCaG were added to 0·45 ml of the same buffer containing 80 μm cytochrome c, 2 mM NaN_3 and the stimulant. After 60 min of incubation at 37°, 0·35 ml from each well was diluted in 0·7 ml of cold PBS and, after centrifugation, the O_2^- produced was quantified from the spectophotometric measurement of the cytochrome c reduced. Monocytes were assayed, immediately after isolation, in plastic tubes agitated in a shaking water bath at 37°. 5×10^5 mononuclear blood cells isolated as described above and suspended in KRPCaG were incubated in 0·5 ml of the same buffer containing 80 μM ferricytochrome c, 2 mM NaN_3 and the stimulant. After 20 min the reaction was stopped by adding 1 ml of cold PBS, and O_2^- calculated by the spectophotometric measurement of cytochrome c reduction in the supernatant. As stimulant, phorbol myristate acetate (PMA) was used routinely. Stock solutions of 2 mg/ml in dimethylsulphoxide, stored at $-20°$ were diluted in KRP and used at a final concentration of 100 ng/ml. In some experiments, zymosan, prepared as described elsewhere (Berton & Gordon, 1983), was

Table 1. Superoxide anion production by AM of sacroid patients assayed immediately after the isolation

	Lymphocytes			nmoles O_2^-/60 min/2·0 × 10^5 AM		
	(%)	No. (× 10^{-3}/ml)	^{67}Ga uptake	CD4/CD8	Resting	PMA (100 ng/ml)
Active sarcoidosis						
G.A.	31	89	++	7·7	3·0	9·9
F.S.	32	70	+++	15·4	4·3	9·6
L.P.	55	258	+++	9·6	2·1	8·6
P.A.	35	52	++	2·2	1·7	11·3
P.R.	35	40	+++	9·5	1·4	8·6
T.P.	37	160	ND	7·5	1·3	3·2
P.I.	47	110	+++	3·5	1·0	8·8
B.G.	32	80	ND	1·9	0·1	3·1
				Mean ± SD:	1·9 ± 1·3	7·9 ± 3·0
Inactive sarcoidosis						
B.R.	5	8	ND	0·9	0·5	2·0
F.D.	7	7	ND	2·3	0·7	3·6
G.L.	5	7	ND	1·4	1·1	5·0
G.A.	8	6	ND	1·7	1·1	2·7
C.M.	10	10	ND	2·3	1·7	2·8
F.V.	5	4	Neg.	0·6	2·5	4·8
S.A.	17	18	Neg.	1·0	5·4	7·0
R.G.	4	6	Neg.	2·5	0·1	1·2
T.D.	4	24	Neg.	1·6	0·4	4·0
				Mean ± SD:	1·5 ± 1·6 (NS)	3·7 ± 1·8 ($P < 0·01$)

For O_2^- production the mean reults of duplicate assays are reported.
ND, dot determined.
NS, not significant.

used as a alternative. H_2O_2 was assayed by the fluorimetric measurement of the horseradish peroxidase-dependent oxidation of homovanilic acid to the fluorescent compound 2,2′-dihydroxy-3,3′-dimethoxydiphenil-5,5′-diacetic acid as described (Valletta & Berton, 1987) in studies with mouse macrophage monolayers. Proteins were assayed as described elsewhere (Berton & Gordon, 1983).

Analysis of data

Values are reported as means ± SD deviation. Significance was determined by the non-paired Student's *t*-test. *P* values > 0·05 are reported in the tables as not significant.

RESULTS

Table 1 shows O_2^- production by AM obtained from BAL of sarcoid patients and assayed immediately after isolation. The data of patients who had a low- or high-intensity lymphocytic alveolitis (see the Materials and Methods) are reported. In the group of patients considered, when performed ^{67}Ga uptake was positive in the group of active and negative in that of inactive sarcoid patients. In the former group the ratio CD4-positive/CD8-positive lymphocytes was always elevated and, apart from two cases, above 2·5; in inactive sarcoid patients the CD4/CD8 ratio was always below this value. AM also produced a low but consistent amount of O_2^- spontaneously, and this was probably due to adherence and spreading to the tissue culture plastic used for the incubation of the cells (Berton & Gordon, 1983). This unstimulated production was comparable in AM of active and inactive sarcoid patients.

PMA at 100 ng/ml stimulated the O_2^- production by AM. Comparison of the mean values of the PMA-stimulated O_2^- production shows that in AM of active sarcoid patients this was two times higher than in AM of inactive sarcoid patients; this difference is statistically significant. These results did not depend on the amount of PMA used since 100 ng/ml was the maximally stimulatory concentration and comparable results were obtained with 500 ng/ml (not shown).

Since we were interested in the oxygen metabolism of AM maintained in culture as adherent cells, we first established that cultivation for a few days did not alter the capability to produce O_2^-. In response to PMA (100 ng/ml), AM of inactive sarcoid patients produced 3·6 ± 1·7 nmoles O_2^-/60 min/2 × 10^5 cells when assayed immediately after the isolation and 3·8 ± 2·2 nmoles O_2^-/60 min/2 × 10^5 cells ($n = 5$) when assayed after 48 hr of culture. With AM of active sarcoid patients, the PMA-stimulated O_2^- production was 6·0 ± 2·5 and 8·0 ± 3·2 nmoles/60 min/2 × 10^5 cells ($n = 4$) when assayed immediately after the isolation or after 48 h in culture, respectively.

Table 2 shows data of O_2^- production by AM of sarcoid patients after 2 days of cultivation. Apart from one with active and four with inactive sarcoidosis, these data were obtained

Table 2. Superoxide anion production by AM after 2 days of cultivation

	Lymphocytes				nmoles O_2^-/60 min /mg protein		/$2{\cdot}0 \times 10^5$	
	(%)	No. ($\times 10^{-3}$/ml)	^{67}Ga uptake	CD4/CD8	Rest.	PMA	Rest.	PMA
Active sarcoidosis								
T.P.	43	113	+++	ND	5·7	79·8	0·3	3·6
F.L.	68	340	++++	ND	0·1	50·7	0·04	2·4
Z.C.	29	182	++++	1·7	4·5	71·0	0·3	4·7
C.L.	54	364	++++	ND	4·1	130·9	0·3	9·6
M.F.	68	209	++++	ND	3·5	92·8	0·3	8·0
C.E.	65	160	++	1·8	19·6	104·8	1·7	13·8
D.P.	47	72	++	3·6	7·3	94·2	1·0	13·0
M.A.	55	141	+	ND	8·1	54·9	0·9	6·2
R.S.	40	92	++	ND	4·0	24·1	0·4	2·4
T.G.	39	111	ND	3·1	18·2	110·9	2·5	15·3
C.D.	48	166	+++	ND	4·6	83·0	0·4	7·2
T.E.	24	70	++++	8·0	4·4	64·1	0·6	8·2
C.D.	51	110	++++	11·6	15·0	53·4	0·8	3·0
C.B.	60	411	++++	17·2	6·1	27·8	0·9	4·0
M.G.	39	78	ND	14·5	8·4	87·1	1·1	11·1
P.I.	47	110	+++	3·5	7·1	101·8	0·8	11·4
Inactive sarcoidosis								
B.L.	5	8	Neg.	ND	0·1	28·6	0·007	2·1
C.V.	4	4	Neg.	2·0	0·1	19·5	0·01	1·9
D.A.	12	10	Neg.	ND	ND	22·4	ND	0·7
D.R.	6	7	Neg.	ND	0·1	10·5	0·006	0·6
P.M.	5	12	Neg.	ND	1·7	30·7	0·1	1·6
R.F.	7	11	Neg.	ND	13·1	88·3	0·6	3·8
P.G.	7	19	Neg.	ND	0·1	77·1	0·01	6·8
M.A.	9	6	Neg.	2·6	4·3	67·4	0·3	5·5
V.E.	8	8	Neg.	1·4	11·1	75·6	0·4	3·2
R.T.	10	10	Neg.	ND	6·6	44·4	0·2	1·6
Z.M.	5	14	Neg.	1·2	7·0	82·7	0·5	6·3
F.V.	5	4	Neg.	0·6	7·1	26·2	0·7	2·6
S.A.	17	18	Neg.	1·0	3·2	11·1	0·4	1·4
R.G.	4	6	Neg.	2·5	0·1	47·9	0·07	3·4
T.D.	4	24	Neg.	1·6	0·1	41·9	0·08	3·6
Healthy subjects								
C.P.	7	6	ND	ND	4·7	23·6	0·1	0·6
C.M.	5	3	ND	ND	1·2	6·2	0·05	0·4
M.A.	2	4	ND	ND	9·3	30·6	0·4	1·2
M.C.	7	5	ND	ND	5·2	15·0	0·2	0·5

AM were isolated from BAL and cultivated for 2 days as described in the text. For O_2^- production the mean results of duplicate assays are reported.

from patients different to those of Table 1. The O_2^- production by AM of healthy subjects is reported for comparison.

The means ± SD of the individual experiments reported in Table 2 are shown in Table 3. Both the values expressed on the basis of the actual amount of adherent proteins and of the number of AM originally plated are reported. Although AM adhered firmly to the plastic used for cultivation, and the detachment after 2 days in culture was negligible, we could not quantify the actual number of cells.

O_2^- production per number of AM, however, gave results comparable to those calculated on the basis of the actual amount of adherent proteins and we report the data for comparison.

The spontaneous O_2^- production by AM after 2 days of cultivation was negligible indicating that, as also shown with mouse peritoneal macrophages (Berton & Gordon, 1983), the stimulation induced by adhesion and spreading (Table 1) was transient.

The production of O_2^- was effectively stimulated by PMA but to a different extent in the three populations tested. AM of

Table 3. Superoxide anion production by AM after 2 days of cultivation

	nmoles O_2^-/60 min/2·0 × 10^5 AM	
	Resting	PMA (100 ng/ml)
Inactive sarcoidosis	0·2±0·2	3·0±1·9
	(14)	(15)
Active sarcoidosis	0·8±0·6	7·7±4·2
	(16)	(16)
Healthy volunteers	0·2±0·1	0·7±0·3
	(4)	(4)
	nmoles O_2^-/60 min/mg proteins	
Inactive sarcoidosis	3·9±4·4	44·9±26·8
Active sarcoidosis	7·5±5·4	77·0±29·7
	NS	$P<0·001$
Healthy volunteers	5·1±3·3	18·8±10·6
	NS	$P<0·001$

The data show mean values±SD of the number of experiments shown in parenthesis and reported in Table 2 individually. Significance was determined by the non-paired Student's *t*-test comparing the O_2^- prodction by AM of active sarcoid patients and healthy subjects with the O_2^- production by AM of inactive sarcoid patients.

NS, not significant.

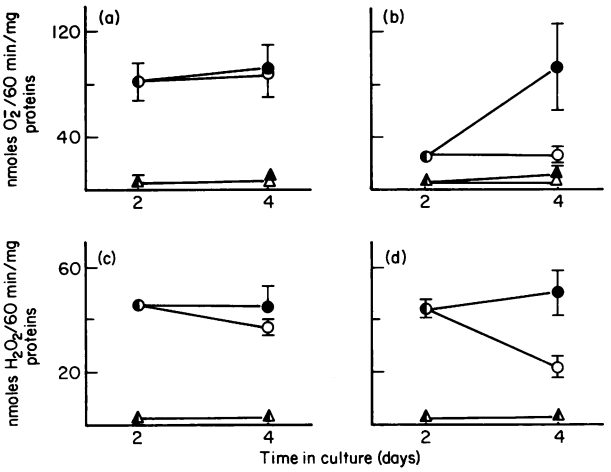

Figure 1. Effect of rIFN-γ on the O_2^- and H_2O_2 production by AM of sarcoid patients. rIFN-γ was added at 50 U/ml at Day 2 of culture and the activity assayed in control (open symbols) and rIFN-γ-treated AM (closed symbols) after 2 further days of cultivation. Triangles: unstimulated activity. Circles: activity in response to 100 ng/ml PMA. Mean results of three independent experiments are shown. (a) and (c): active sarcoid patients. (b) and (d): inactive sarcoid patients.

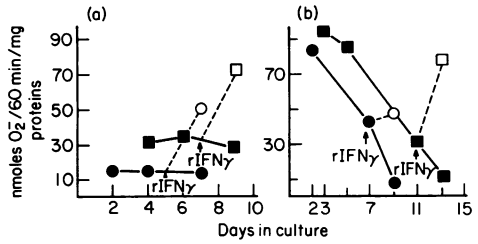

Figure 2. Effect of rIFN-γ on the O_2^- production by AM of sacroid patients after prolonged cultivation. rIFN-γ was added at 50 U/ml at the times indicated by the arrows. (a) Shows results obtained with inactive and (b) with active sarcoid patients. Closed symbols: activity of control cells. Open symbols: activity of rIFN-γ-treated cells. In the experiment represented by circles in (b), after 24 hr of cultivation in rIFN-γ-containing medium, AM were washed and maintained in rIFN-γ-free medium for a further 24 hr before the assay. O_2^- production was stimulated with 100 ng/ml PMA and unstimulated activity was < 10% of the stimulated one (not shown). Mean results of duplicate assays which varied < 10% are reported.

sarcoid patients produced more O_2^- in response to PMA than control AM and this difference was consistent and significant. In sarcoid AM, the production of O_2^- in response to PMA appeared to be correlated with the state of the disease. AM isolated from the BAL of patients with active sarcoidosis produced more O_2^- in response to PMA than AM of patients with inactive sarcoidosis and the difference was significant. Similar results were obtained also with another stimulant of oxygen metabolism like zymosan. This was less efficient than PMA as a triggering agent (three to five times enhancement above the background) and stimulated the production of 1·1±0·3 ($n=4$) and 2·7±0·4 ($n=4$) nmoles O_2^-/60 min/2 × 10^5 cells in AM of patients with inactive or active sarcoidosis, respectively.

O_2^- production by active sarcoid patients was accompanied by the production of H_2O_2 in amounts stoichiometric with the theoretical value of 2 moles of O_2^- per mole of H_2O_2. In five independent experiments where O_2^- and H_2O_2 production were assayed in parallel, AM of patients with active sarcoidosis produced 108·9±36·3 nmoles O_2^-/60 min/mg proteins and 55·9±5·0 nmoles H_2O_2/60 min/mg proteins.

IFN-γ released by activated T lymphocytes has been recognized as the molecule responsible for the activation of human monocyte-derived (Nathan *et al.*, 1983) and alveolar (Murray *et al.*, 1985; Fels, Nathan & Cohn, 1987) macrophage oxygen metabolism. Since we had an indication that the enhanced capability to produce O_2^- by AM in active sarcoidosis could be correlated with the presence of T lymphocytes in the alveolus, we asked whether IFN-γ, which is released by mononuclear cells present in the alveolus of sarcoid patients (Robinson *et al.*, 1985; Nugent *et al.*, 1985), could activate AM to produce higher amounts of O_2^-

Figure 1 shows the data we obtained with AM of active and inactive sarcoid patients. In (a) it is shown that AM of patients with active sarcoidosis, which produced high amounts of O_2^- in response to PMA after 2 days of cultivation, maintained this capability up to Day 4 of culture and rIFN-γ was not able to enhance it. Similar results were obtained by assaying H_2O_2 production.

As shown in (b), AM of inactive sarcoid patients, which produced low amounts of O_2^- in response to PMA either at Day 2 or 4 of culture were, however, responsive to rIFN-γ which enhanced considerably the capability to produce O_2^- after 2 days of treatment. Comparable results were obtained in assays of H_2O_2 production (d): in this case, the AM tested had an activity similar to AM of active sarcoid patients after 2 days of culture, but this declined at Day 4; inclusion of rIFN-γ in the culture medium prevented this decline.

Table 4. PMA-stimulated superoxide anion production by monocytes isolated from blood of sarcoid patients and healthy volunteers

nmoles O_2^-/20 min/10^6 monocytes		
Control	Active sarcoidosis	Inactive sarcoidosis
42·8±21·5 (6)	41·0±2·9 (5)	35·0±3·5 (2)

Mononuclear white blood cells were isolated by centrifugation over Ficoll–Paque. Spontaneous O_2^- production in the absence of PMA (100 ng/ml) was < 5 nmoles/20 min/10^6 monocytes. Number of monocytes was quantified by non-specific esterase staining.

Table 5. PMA-stimulated superoxide anion production by monocytes and monocyte-derived macrophages of normal subject and sarcoid patients. Effect of cultivation

	nmoles O_2^-/60 min/mg proteins		
Days in culture	2	7	11
Control	156·7±32·8 (3)	87·5±13·3 (3)	12·7±7·5 (3)
Active sarcoidosis	161·2±34·6 (4)	93·7±29·7 (4)	9·7±3·6 (4)

Monocytes were isolated by centrifugation over Ficoll–Paque and cultivated for different days. Spontaneous O_2^- production in the absence of PMA (100 ng/ml) was < 10% of the PMA-stimulated activity. The data shows means±SD of the number of experiments reported in brackets.

Figure 2 shows that also AM of patients with active sarcoidosis could be activated *in vitro* by rIFN-γ to produce higher amounts of O_2^- in response to PMA. In the two experiments reported, production of O_2^- was high after 2 days of cultivation, but declined during maintenance in culture. Addition of rINF-γ when the capability to produce O_2^- had declined to low levels boosted it again or prevented further decline. The results obtained with AM of inactive sarcoid patients cultivated for longer times are shown for comparison.

We were interested to know if the activation of the macrophage capability to metabolize oxygen in sarcoidosis was a local phenomenon or was also reflected by an enhanced activity of circulating monocytes. Tables 4 and 5 show data obtained with monocytes assayed immediately after isolation of mononuclear white cells or after different times of cultivation. These data clearly indicate that the capability to produce O_2^- in response to PMA by circulating monocytes and the time–course of its down-modulation in culture are not different in patients with sarcoidosis compared with controls.

DISCUSSION

The results presented in this paper show that in patients with sarcoidosis, AM are activated in their capability to metabolize oxygen with production of superoxide anion. In the active state of the disease, when T lymphocytes accumulate in the alveolus, this capability is enhanced in comparison with the inactive state when alveolitis is of low intensity.

AM of sarcoid patients produced consistent amounts of O_2^- spontaneously if assayed immediately after the isolation. This is in accordance with previous observations (Fels *et al.*, 1987; Aerts *et al.*, 1986) and can be due to adherence and spreading to plastic surfaces; production of O_2^- during spreading is a feature of activated mouse peritoneal macrophages (Berton & Gordon, 1983). As in mouse macrophages, the spontaneous production of O_2^- by AM was transient and negligible after 2 days of cultivation.

The stimulation of oxygen metabolism with PMA allowed us to reveal that the capability to metabolize oxygen is activated in sarcoid macrophages and correlates with the extent of the lymphocytic alveolitis. The enhanced production of O_2^- in response to PMA by AM of active sarcoid patients was observed either with freshly explanted cells or with cells cultivated for 2 days, indicating that it is not a rapidly transient phenomenon.

The enhanced production of O_2^- by AM of sarcoid patients and, among these, its dependence on the extent of the lymphocytic alveolitis can be explained as a result of either the release of factor(s) able to activate macrophages resident in the alveolus or the recruitment of monocytes from the circulation.

Evidence has been presented that IFN-γ released by activated T lymphocytes is the cytokine responsible for the activation of macrophage capability to metabolize oxygen with production of O_2^- and H_2O_2 (Nathan *et al.*, 1983, 1984). IFN-γ is spontaneously released by lung mononuclear cells present in the alveolus of sarcoid patients in culture, and cells of patients with active sarcoidosis release higher amounts of IFN-γ than those of patients with inactive sarcoidosis (Robinson *et al.*, 1985). As also previously reported (Murray *et al.*, 1985, Fels *et al.*, 1987), the data presented in this paper show that IFN-γ can activate AM. Significantly, AM of patients with active sarcoidosis which were already activated to produce high amounts of O_2^- did not respond to IFN-γ with a further enhancement of this capability. As with monocytes differentiated to mature macrophages by *in vitro* cultivation, the capability to produce O_2^- by AM of active sarcoidosis patients could be down-modulated by prolonged maintenance in culture, and this was accompanied by the recovery of the sensitivity to an IFN-γ challenge. These data do indeed suggest that the oxygen metabolism of tissue resident macrophages can be activated by IFN-γ and that macrophages can alternate states of high or low activity in dependence on the local environment.

Another reason for the enhanced capability to produce O_2^- expressed by macrophages isolated from the lung of patients with active sarcoidosis could be the accumulation in the alveoli of monocytes recruited from the circulation. Evidence has indeed been presented that the enhanced expression of monocyte surface antigens in AM of active sarcoidosis patients is likely due to recruitment of monocytes from the blood and not to phenotypic changes induced in the population resident in the alveolus (Hance *et al.*, 1985; Agostini *et al.*, 1987). A monoclonal antibody that reacts only with LPS- or IFN-γ-activated

monocytes and macrophages (Ewan *et al.* 1986), however, was shown to bind to all the macrophages present in the BAL of patients with active sarcoidosis (Hancock *et al.*, 1986), indicating that local release of activating factors must definetively occur.

We do not know the percentage of macrophages derived from recently emigrated monocytes in our system. However, in our AM monolayers the cells did indeed appear as large, mature macrophages, well distinct from circulating monocytes. Moreover, the amount of adherent proteins per plated cells in AM of active sarcoidosis patients was the highest among the populations studied (see the Materials and Methods).

The data shown in Tables 4 and 5 indicate that the activation of the capability to metabolize oxygen in sarcoidosis is likely a local phenomenon and is not accompanied by activation of monocytes in the circulation. Although circulating monocytes already produce high amounts of O_2^- (Nagakawara *et al.*, 1981; Pabst *et al.*, 1982; Cassatella *et al.*, 1985), this capability is enhanced by *in vitro* treatment with rINF-γ (Nathan *et al.*, 1983; Weinberg, Hobbs & Misukonis, 1984) or in cancer patients who received intravenous rIFN-γ (Nathan *et al.*, 1985). However, monocytes isolated from blood of sarcoid patients produced amounts of O_2^- comparable to control values.

ACKNOWLEDGMENTS

This work was supported by grants from Consiglio Nazionale delle Ricerche, Gruppo Immunologia (n.85.00720.04 and 86.00478.04) from Ministero Pubblica Instruzione (fondi 40%) and from University of Padova (n.12.01.4689).

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