Immunology 1988 **64** 281–288

Mechanisms by which oxidative injury inhibits the proliferative response of human lymphocytes to PHA. Effect of the thiol compound 2-mercaptoethanol

M.-A. GOUGEROT-POCIDALO, M. FAY, Y. ROCHE & S. CHOLLET-MARTIN *INSERM U. 294 and Laboratoire d'Immunologie et d'Hématologie, Chu Bichat, Paris, France*

Accepted for publication 1 February 1988

SUMMARY

The use of normobaric exposure to O_2 as a model for *in vitro* oxidative injury prevented phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMC) from undergoing the G0 to G1 transition, but 5×10^{-6} M 2-mercaptoethanol (2-ME) almost protected the cells from this blockade. The percentage of cells with IL-2 and transferrin-receptors was reduced by the O_2 exposure and, like the cell cycle transition, was protected by 2-ME against oxidative injury. By contrast, IL-2 recovery in the supernatants of O_2-exposed PHA-stimulated PBMC was enhanced. This enhancement may be due partly to the reduced IL-2 consumption caused by the decreases in IL-2 receptor expression and in proliferation. On the other hand, IL-2 recovery in the supernatants of O_2-treated PBMC was always enhanced compared to the IL-2 control recovery after DNA synthesis was blocked in G1/S by mitomycin *c*, and the G0/G1 transition was protected by 2-ME. Furthermore, PHA-stimulated monocytes exposed to O_2 produced more IL-1 than control cells. This enhanced IL-1 production was not modified by 2-ME. These results suggest that oxidative injury reduces the proliferation of PBMC by interfering with the cellular events that lead to the transition from the G0 to the G1 phase of the cell cycle. The protective effects of 2-ME suggest that thiol compounds have a critical role in the early events of the cell cycle. By contrast, exposure to O_2 induced increases in the production of both IL-1 and IL-2 that may not be related to alterations in the thiol status of the cell.

INTRODUCTION

Immune depression induced by species derived from oxygen (O_2) has been incriminated in several physiological and pathological conditions (Harman, Heidrick & Eddy, 1977; Doria, Agarossi & Adonini, 1982; Trush, Minnaugh & Gram, 1982). Furthermore, during inflammatory processes, phagocytes constitute a major delivery system for these O_2-derived species, which might induce tissue injury (Henson & Johnston, 1987) and also influence lymphocyte functions (Sagone, Kamps & Campbell, 1978). In reports from this laboratory, it has been demonstrated previously that normobaric exposure to O_2 is an appropriate model of immune oxidative injury *in vivo* (Levacher-Place *et al.*, 1983; Gougerot-Pocidalo *et al.*, 1985) and *in vitro* (Kraus *et al.*, 1985). In particular, it has been shown that splenic cells from mice exposed to normobaric O_2 *in vivo* and immune cells from rats similarly exposed *in vitro* have a depressed proliferative response to mitogens. These findings were in accordance with the observations made using other *in vitro* oxidative processes, such as enzymatic systems that generate O_2-derived products (Hoffeld, Metzger & Oppenheim, 1981) or oxidizing reagents (Chaplin & Wedner, 1978; Fishman *et al.*, 1981). In humans, normobaric O_2 exposure or enzymatic systems that generate O_2-derived products were found, *in vitro*, to inhibit the mitogenic response of peripheral blood mononuclear cells (PBMC) (Sagone *et al.*, 1978; Karlberg *et al.*, 1981).

The biochemical mechanisms associated with oxidative immune depression are partially known and the role of the cellular sulphhydryl compounds in this depression has been investigated. In mice and rats, the proliferative response that is depressed both by oxidizing reagents and normobaric O_2 is partially protected by the addition of thiol compounds to the cultures, especially 2-mercaptoethanol (2-ME) (Noelle & Laurence, 1981a, b; Gougerot-Pocidalo, Fay & Pocidalo, 1984; Gougerot-Pocidalo *et al.*, 1985; Kraus *et al.*, 1985), whose role in maintaining the thiol status of the cell is well established (Zmuda & Fridenson, 1983; Ohmori & Yamamoto, 1982, 1983). However, as far as we know, the effect of thiol compounds such

Abbreviations: IL-, interleukin; 2-ME, 2-mercaptoethanol; O_2, oxygen; PBMC, peripheral blood mononuclear cells.

Correspondence: Dr M.-A. Gougerot-Pocidalo, Laboratoire d'Immunologie & d'Hématologie, Chu Bichat, 46 rue Henri Huchard, 75877 Paris, Cedex 18, France.

as 2-ME on oxidative-injured human lymphoid cells has never been investigated. The aim of this work was (i) to analyse cellular activation events that would be perturbed by injury from exposure to O_2 and would lead to the depression of proliferation and (ii) the effect of 2-ME on these events. Therefore, we cultured PHA-stimulated human PBMC in an O_2 or normal air atmosphere, in the presence or absence of 2-ME, and studied the cell cycle progression, the expression of receptors for interleukin-2 (IL-2) and transferrin, as well as IL-2 production.

MATERIALS AND METHODS

Culture medium and reagents

The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) supplemented with 25 mM HEPES, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 100 mM pyruvate, 2 mM glutamine, and 1% non-essential amino acids (Gibco). Fetal calf serum (FCS) (Boehringer, Mannheim, FRG) was heat-inactivated and kept frozen at −30° until use. The reagents used were the following: 2-mercaptoethanol (2-ME) (Merck, Darmstadt, FRG); phytohaemagglutinin (PHA) (Wellcome, Beckenham, England); methyl-[^{3}H]thymidine ([^{3}H]TdR; specific activity 2 Ci/mmol; CEA, Saclay); recombinant IL-2 (Genzyme, Tebu), and mitomycin *c* (Choay, Paris).

Exposure conditions

Cultures were incubated at 37° in a humidified normobaric oxygen atmosphere in a leak-proof chamber, as described previously (Kraus *et al.*, 1985). This incubation under 95% O_2 and 5% CO_2 lasted for 16, 24 or 48 hr. It was then extended, under 95% air and 5% CO_2, to a total of 24, 48 or 72 hr, which corresponded to the incubation periods for control cultures. The latter were continuously incubated under 95% air and 5% CO_2.

PBMC preparation and cultures

Human PBMC were isolated from heparinized blood of healthy adult donors by the method of Böyum (1968) on a Ficoll–Isopaque gradient (Pharmacia, Uppsala, Sweden). The cells were then washed and resuspended in RPMI-1640 medium. In some experiments, PBMC were pretreated by incubation for 30 min at 37° with 40 μg/ml of mitomycin *c*, washed three times and suspended in culture medium. PBMC were adjusted to 10^6 viable cells/ml in culture medium supplemented with 5% FCS. Cultures were set up in microtest plates (Falcon, Microtest III, Oxnard, CA). Cells, 10^5, were incubated for 72 hr in 200 μl culture medium with or without 1 μg/ml PHA. These cultures were supplemented with 20 μl of culture medium, or with 2-ME in culture medium at a predetermined optimal concentration of 5×10^{-6} M. The plates were incubated at 37° in a humidified atmosphere of either 95% air and 5% CO_2 (control air) or 95% O_2 and 5% CO_2 (normobaric O_2) for 16, 24 or 48 hr, as described above. Cell viability was determined by trypan blue exclusion or ethydium bromide.

[^{3}H]TdR incorporation

Each culture was done in triplicate to permit the assay of cell proliferation, and each well was pulsed with 1 μCi[^{3}H]TdR for the last 6 hr of the 72-hr culture period. Cells were harvested on glass fibre filters with a multiple cell culture processor (Skatron, A.S. Lier, Norway). [^{3}H]TdR incorporation was counted with a liquid scintillation spectrometer (SL 3000, Intertechnique, Montigny) and expressed as counts per minute (c.p.m.). The percentage of proliferation was considered as the ratio of the c.p.m. for [^{3}H]TdR incorporation of PBMC cultured under normobaric O_2, as defined above, to the c.p.m. obtained under 95% air and 5% CO_2.

Cell cycle analysis

The effect of O_2 exposure on the PHA-induced activation and proliferation of human lymphocytes was assessed by the method of Darzynkiewicz *et al.* (1979). PHA-stimulated cells, cultured as described above, were made permeable by adding 200-μl aliquots containing 2×10^6 cells to 400 μl of a chilled solution containing 0·1% Triton X-100 (Sigma, St Louis, MO), 0·08 N HCl and 0·15 M NaCl. Thirty seconds later, the cells were stained by adding 1·2 ml of a solution containing 25 μg/ml purified acridine orange (Polysciences Inc., Warrington, PA), 0·04 M citric acid, 0·12 M Na_2PO_4, 0·1 M NaCl, and 10^{-3} M EDTA-Na (Merck). The red (RNA) and green (DNA) fluorescence emissions from each cell were analysed in a system 50 HH cytofluorograph (Orthodiagnostic Instruments Westwood, MA) equipped with a model 2150 computer. The results were based on the analysis of 30×10^3 cells per sample, excluding dead cells and doublets. Regions containing cells in G0 were determined using freshly prepared lymphocytes. The boundary between the G1 and S-phase regions was determined with PHA-stimulated lymphocytes to which 2 mM hydroxyurea (Sigma, St Louis, MO) was added at the initiation of the culture. In control experiments, Vinblastine (Sigma) at a final concentration of 0·2 μg/ml, was added for the final 24 hr of culture to prevent the return of cells undergoing mitosis to G0.

Fluorescent cell staining

After 48 or 72 hr culture, 10 wells of PBMC were harvested for each culture condition as described above, pooled and washed once with PBS containing 5% FCS and 0·2% sodium azide (PBS-Az; Mérieux, Charbonniers). Indirect immunofluorescence was then carried out as described earlier (Chollet-Martin & Gougerot-Pocidalo, 1986) by incubating 5×10^6 cells on ice for 30 min with appropriate dilutions of one of the following monoclonal antibodies: anti-IL-2 receptor (CD 25), anti-Transferrin receptor (Becton-Dickinson, Mountain View, CA), IOT3 (CD3), IOT4 (CD4), and IOT8 (CD8) (Immunotech, Luminy, Marseille). Background fluorescence was determined with diluted normal mouse serum instead of the first antibody. Cells were washed twice with PBS-Az, and reincubated for 30 min on ice with fluorescein-labelled goat anti-mouse antibodies (GAM-FITC, Nordic Laboratories, Tilburg, The Netherlands) at a final dilution of 1/20. Cells were washed twice again before analysis by the Ortho cytofluorograph using an argon ion laser at an excitation wavelength of 488. Non-viable cells were gated out using ethidium bromide; 10,000 cells were accumulated for each histogram and the percentage of positive cells was obtained by computed histograms. Background fluorescence was subtracted from all samples.

Production of supernatants containing IL-2 and assay for IL-2 activity

Supernatants from quadruplicated wells, performed under the same conditions as the proliferation, were harvested after various periods of incubation and atmospheric exposure. The

Table 1. Effect of exposure to normobaric O_2 on [^{3}H]TdR incorporation by PHA-stimulated human PBMC

Addition* to culture	[^{3}H]TdR† incorporation	Control air‡	O_2 exposure time§ (hr)		
			16	24	48
Medium	C.p.m. $\times 10^{-3}$¶	64·98 ± 4·34	53·73 ± 6·32	26·17 ± 2·24	6·02 ± 1·10
	O_2/air**	100	80·27 ± 5·45	41·11 ± 4·40	9·30 ± 2·07
2-ME	C.p.m. $\times 10^{-3}$¶	72·20 ± 6·59	72·59 ± 2·11	58·84 ± 4·05	17·09 ± 1·67
	O_2/air**	100	99·05 ± 5·50	80·28 ± 5·84	23·80 ± 2·18

* At the initiation of the cultures, the medium was supplemented with either 20 μl of the same medium or 2-ME diluted in the medium to obtain a final concentration of 5×10^{-6} M 2-ME.

† Human PBMC, 10^5, were incubated in the presence of PHA at a final concentration of 1 μg/ml, as described in the Materials and Methods.

‡ Cells were cultured for 72 hr under 95% air and 5% CO_2.

§ Cells were cultured for various periods, first under 95% O_2 and 5% CO_2, and then under 95% air and 5% CO_2, until a total of 72 hr culture was obtained.

¶ Cells were pulsed with 1 μCi[^{3}H]TdR 6 hr before the end of culture. Results are means ± SEM of seven experiments and are expressed as c.p.m. $\times 10^{-3}$. Incorporation of [^{3}H]TdR by non-stimulated cells was always less than 250 c.p.m.

** The percentage of proliferation, expressed as O_2 c.p.m./air c.p.m. was calculated by dividing c.p.m. [^{3}H]TdR incorporation of the O_2 exposed cells by the corresponding value for control air-exposed cells × 100.

supernatants were pooled, centrifuged at 800 ***g*** for 10 min and frozen at −20° until they were assayed for IL-2 activity, as described previously (Gillis *et al.*, 1978), using the murine IL-2-dependent cell line CTLL-2 (Institut Pasteur, Paris). Briefly, cells were washed and an 0·1-ml aliquot containing 1×10^4 cells was mixed with 0·1 ml of serial dilutions of the test sample. After 18 hr incubation, cultures were pulsed for 6 hr with 1 μCi of [^{3}H]TdR and the radioactivity incorporated was determined as described above. IL-2 titres in the culture supernatant were determined by comparison with the activity of appropriately diluted human recombinant IL-2 (R IL-2, Genzyme) using a computer program (provided by courtesy of Dr Alan Hance, INSERM, Paris) and were expressed as units IL-2/ml.

Monocyte cultures and IL-1 production

PBMC monocytes were counted by non-specific esterase staining, adjusted in RPMI-1640 medium with 1% FCS to a concentration of $0{\cdot}2 \times 10^6$ monocytes per ml and incubated in multiwell plastic plates (24 × 17 mm, Nunclon, Denmark) as reported previously (Roche, Fay, Gougerot-Pocidalo, 1987). After removing the non-adherent cells, culture medium without PHA, or with 1 μg/ml PHA (final concentration), was added to each well. Cultures were then incubated for another 24 or 48 hr in the air or oxygen environments specified above. Extracellular and cell-associated IL-1 activities were determined in the cell-free supernatants and in the cellular lysates, respectively. IL-1 activity was considered as the capacity of IL-1 to stimulate thymocyte proliferation in the presence of a submitogenic concentration of PHA, as described by Scala & Oppenheim (1983). Accordingly, aliquots of 0·1 ml of the diluted supernatants or lysates to be assayed and 0·1 ml of the thymocyte suspension diluted 15×10^6 cells/ml in RPMI-1640 containing 1 μg/ml PHA, were incubated for 72 hr at 37° in a 5% CO_2 incubator, and 1 μCi of [^{3}H]TdR was added to each well 6 hr before the end of incubation. Cells were harvested and radioactivity was counted as described above. The c.p.m. for the samples from monocytes stimulated by PHA under air or O_2 conditions were corrected for background c.p.m., i.e. the control medium handled in the same way. Assay of CTLL-2 cells for IL-2 did not reveal detectable IL-2 activity in the supernatants.

Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test. All values were expressed as means ± SEM.

RESULTS

Effect of *in vitro* exposure to normobaric O_2 on PHA-induced [^{3}H]TdR incorporation by PBMC

As shown in Table 1, PHA-induced [^{3}H]TdR incorporation by PBMC decreased in a time-dependent manner as exposure to O_2 lengthened.

The effect of this exposure on the number of viable cells was determined after 72 hr culture on non-stimulated PBMC, to avoid taking into account the effect of the oxidative injury on cell proliferation. As shown in Table 2, exposure of unstimulated PBMC to normobaric O_2 for 24 hr did not alter cell viability significantly. However, 48 hr exposure significantly reduced the number of viable cells to 81% of the number in control cells. Nevertheless, this decrease in viability was slight compared to the drop in proliferation observed under the same conditions.

Protection by 2-ME against the reduced proliferation caused by oxidative injury

When the optimal concentration of 5×10^{-6} M 2-ME, determined from control cell proliferation, was added to PHA-stimulated PBMC cultures, it afforded significant protection against the reduction of proliferation caused by exposure to O_2

Table 2. Effect of exposure to normobaric O_2 on the viability of human PBMC

Addition to† culture	Number of viable cells × 10^{-3}*			
	Control	O_2 exposure time (hr)‡		
	72 hr air‡	16	24	48
Medium	86·17±2·15	85·92±2·64 NS	81·95±2·25 NS	70·02±2·91 §
2-ME	90·02±4·03	92·42±3·33 NS	89·50±7·64 NS	75·82±3·70 ¶

* Cells, 10^5, were incubated per well in the absence of PHA.

† At the initiation of the cultures, the medium was supplemented with either 20 μl of the same medium or 2-ME diluted in the medium to obtain a final concentration of 5×10^{-6} M 2-ME.

‡ Cells were cultured either for 72 hr under 95% air and 5% CO_2 or for various periods under 95% O_2 and 5% CO_2 and then under 95% air and 5% CO_2 until a total of 72 hr culture was obtained. At the end of the culture, viable cells were counted by trypan blue exclusion. Results are means ± SEM of four experiments.

§ $P < 0.01$.

¶ $P < 0.05$.

NS, not significant.

(Table 1). Thus, for cells exposed to normobaric O_2 for 24 hr, the percentage response in terms of O_2 c.p.m./air c.p.m. increased from 41±4% without 2-ME, to 80±6% in the presence of 2-ME ($P < 0.001$). This protection was not due to the improvement of viability by 2-ME, because the numbers of viable cells were not significantly different in its presence or absence, whether cells were cultured in normal air or normobaric O_2 (Table 2).

Effect of normobaric exposure on the cell cycle

Because 24 hr of O_2 exposure approximately halved [^{3}H]TdR incorporation without changing viability, we chose to study cellular events using this period of O_2 exposure.

As shown in Fig. 1d, e and Table 3, the cell cycle analysis after 48 hr of culture confirmed the results obtained by [^{3}H]TdR incorporation. Thus, 24 hr of exposure to O_2 halved the percentage of cells that progressed to the S and G2, M phases. Analysis of the RNA content showed that 17·5% of the cells exposed to O_2 reached the G1 phase, versus 30·0% of those exposed to air. Unlike control cells, the O_2-exposed cells accumulated in G0. The use of Vinblastine to prevent the return to G0 of cells reaching the G2, M phase did not alter the accumulation of O_2-exposed cells in G0 (data not shown). Similar results were observed after 72 hr culture.

Furthermore, Fig. 1f, g and Table 3 show that 2-ME almost protected the cells against the O_2-induced blockade, since it allowed O_2-exposed cells to progress better from G0 to S, decreasing the O_2-induced accumulation of cells in G0.

Effects of normobaric O_2 exposure on the expression of IL-2 and transferrin receptors—protective effect of 2-ME

As shown in Table 4, 24 hr normobaric exposure to O_2 of PHA-stimulated PBMC significantly reduced the expression of the

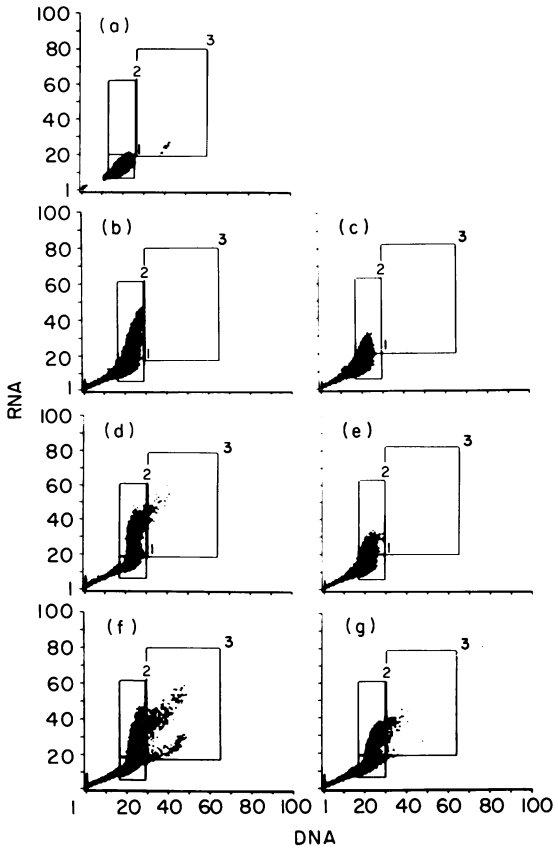

Figure 1. Cell cycle analysis of PHA-stimulated PBMC cultured under air or O_2 conditions. PHA-stimulated PBMC were cultured in RPMI medium (d, e), or in medium containing either 2 mM hydroxyurea (b, c) or 5×10^{-6} M 2-ME (f, g) as described in the Materials and Methods. Cells were cultured either first for 24 hr under 95% O_2 and 5% CO_2 and then for 24 hr under 95% air and 5% CO_2 (O_2 exposed cells in c, e, g), or for the entire 48 hr period under 95% air and 5% CO_2 (control air in b, d, f). Regions were set by using freshly prepared PBMC (a) for G0 and hydroxyurea-treated PHA-PBMC cultured under air conditions were used to set G1/S boundary (b).

activation markers, i.e. IL-2 and transferrin receptors. In addition, the presence of 5×10^{-6} M 2-ME conferred significant protection against these decreases. Similar results were observed after 48 and 72 hr culture.

Effects of normobaric O_2 exposure on IL-2 production by PHA-stimulated PBMC

As shown in Fig. 2a, 24 hr exposure to normobaric O_2 significantly enhanced the IL-2 activity recovered in the cell-free supernatants of PHA-stimulated human PBMC, and this recovery increased with the period of culture.

As shown in Fig. 2b, enhancement of IL-2 activity in the supernatants of cultures exposed to O_2 for 24 hr was significantly lower in the presence of 2-ME than in its absence. Thus, after 48 hr culture, the mean ratios of IL-2 activity ± SEM in the supernatants of cells exposed to O_2 over the activity of cells exposed to air were 3·98±0·70 and 2·62±0·42 ($n = 6$, $P < 0.05$) in the absence and presence of 2-ME, respectively. Because IL-2 is absorbed and consumed by proliferating cells, modifications

Table 3. Cell cycle analysis by regions for Figure 1

Addition to cultures	Exposure conditions	% cells in G0	G1	S+G+M
Medium	Air	43·5	30·0	26·5
	O_2	69·9	17·5	12·6
Hydroxyurea (2 mM)	Air	59·9	33·5	6·6
	O_2	76·7	16·8	6·5
2-ME	Air	43·5	18·3	38·2
	O_2	49·1	20·5	30·4

Legend as explained for Figure 1.

G0 corresponds to region 1, G1 to region 2, S+G2+M to region 3. The percentage of cells in each phase of the cell cycle was determined as described in the Materials and Methods. Three experiments were performed and the results of one representative experiment are shown here.

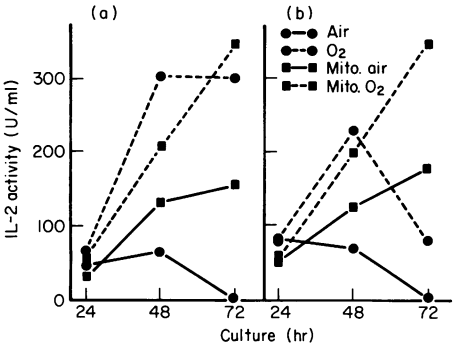

Figure 2. IL-2 activity recovered in the supernatants of peripheral blood mononuclear cells (PBMC) exposed to O_2 (- - -) or air (———) that were pretreated with mitomycin *c* (mito.) (■) or not so (●), as described in the Materials and Methods. At the initiation of the cultures, the medium was supplemented with 20 μl of the same medium (a) or 2-ME diluted in the medium to obtain a final concentration of 5×10^{-6} M 2-ME (b). The PHA-stimulated cells were either cultured first for 24 hr under 95% O_2 and 5% CO_2, and then for various periods under 95% air and 5% CO_2, or under 95% air and 5% CO_2 for the entire culture period. The supernatants were collected after 24, 48 or 72 hr culture. IL-2 activity was measured using the proliferation of CTLL2, as described in the Materials and Methods. Results are expressed as units/ml and represent the mean of triplicate culture of a typical experiment.

Table 4. Effect of exposure to normobaric O_2 on the expression of IL-2 and transferrin receptors

Addition to culture†	Percentage of positive cells with* Anti-IL-2 receptors Air‡	Anti-IL-2 receptors O_2	Anti-transferrin receptors Air	Anti-transferrin receptors O_2
Medium	46·7±1·8	34·3±1·8§	45·3±1·5	33·6±1·2§
2-ME	52·7±3·1¶	47·0±2·7¶	50·4±2·7¶	43·6±2·5¶

* The percentage of PBMC with IL-2 or transferrin receptors after stimulation with 1 μg/ml PHA was determined by indirect immunofluorescence staining (see the Materials and Methods).

† At the initiation of the cultures, the medium was supplemented with either 20 μl of the same medium or 2-ME diluted in the medium to obtain a final concentration of 5×10^{-6} M 2-ME.

‡ Cells were cultured, either first for 24 hr under 95% O_2 and 5% CO_2 and then for 24 hr under 95% air and 5% CO_2 (O_2), or for the entire 48-hr period under 95% air and 5% CO_2 (air). Results are means ± SEM of four experiments.

§ Significant difference ($P < 0{\cdot}02$) between the percentage of positive cells in O_2 and air-exposed cells.

¶ Significant difference ($P < 0{\cdot}02$) between the percentage of positive cells counted in the presence and absence of 2-ME.

in the proliferative response to PHA may explain the above differences in the recovery of IL-2 in the extracellular medium. Therefore, we studied IL-2 activity after pretreating the cells with mitomycin *c*, which blocks the cell cycle at the G1/S interphase. As expected (Stadler *et al.*, 1981) this increased the amount of IL-2 activity recovered after 48 and 72 hr culture under air in the supernatants of PHA-stimulated PBMC (Fig. 2a). However, even though we blocked the cell cycle at the G1/S interphase, IL-2 activity remained higher in the supernatants of cells exposed to O_2 than in those of cells exposed to air. This might be due to the O_2-induced decrease in IL-2 receptor

Table 5. Effect of exposure to normobaric O_2 on IL-1 production by human monocytes*

IL-1 activity† (c.p.m.)	Exposure conditions	Time of culture (hr) 24	48
Supernatant	Air	3081±326	4509±774
	O_2	8129±1026‡	24171±2913§
Cell-associated	Air	8316±1915	4239±1780
	O_2	24213±4220§	9888±1550‡

* Human monocytes stimulated with PHA (1 μg/ml) were cultured for 24 or 48 hr under 95% air and 5% CO_2 (air) or 95% O_2 and 5% CO_2 (O_2).

† IL-1 activity in cell-free supernatants and cellular lysates was evaluated by determining [^{3}H]TdR incorporation by C3H He thymocytes in the presence of a 1:4 dilution of each sample, as described in the Materials and Methods. Results are means ± SEM of three experiments and expressed as c.p.m. $\times 10^{-3}$. The c.p.m. for thymocyte proliferation in response to the cell-free supernatants, and the cellular lysates obtained from unstimulated monocytes were 500±50 and 350±100, respectively.

‡ $P < 0{\cdot}01$.

§ $P < 0{\cdot}001$.

expression. However, addition of 2-ME to the cultures of cells pretreated with mitomycin *c* did not reduce the enhanced recovery of IL-2 in the supernatants of cells exposed to O_2 (Fig. 2b) but did protect the expression of IL-2 receptors in these cells (data not shown). Thus, after 48 hr culture, the mean ratios of IL-2 activity ± SEM in the supernatants of cells exposed to O_2 over the activity in cells exposed to air were $1{\cdot}5 \pm 0{\cdot}1$ and $1{\cdot}6 \pm 0{\cdot}1$ in the absence and presence of 2-ME, respectively ($n = 3$).

Effect of exposure to normobaric O_2 on IL-1 production by human monocytes

In order to assess the increase in the IL-2 activity recovered in the supernatants of PHA-stimulated PBMC exposed to O_2, we studied the effects of this exposure on extracellular and cell-associated IL-1 activity after stimulating human monocytes with 1 μg/ml PHA. As shown in Table 5, exposure to normobaric O_2 increased both the extracellular and cell-associated IL-1 activity of these monocytes, thus enhancing their total IL-1 production. This increased activity was not modified by the addition of 5×10^{-6} M 2-ME at the initiation of culture (data not shown).

DISCUSSION

Our results demonstrate that the use of exposure to normobaric O_2 as an oxidative injury model prevents the transition of PHA-stimulated human lymphocytes from the G0 to the G1 phase of the cell cycle. This blocking effect may explain why O_2 reduced the proliferative response of human PBMC to PHA, as described previously (Karlberg *et al.*, 1981) and as confirmed in this study. Thus, 24 hr exposure to O_2 of PHA-stimulated cells simultaneously induced a decrease of about 50%, both in [^{3}H]TdR incorporation and in the percentages of cells in the S, G2, M and G1 phases, with a concomitant accumulation in the phase G0 (Tables 1 and 3).

Immune consequences of oxidative injury have been well demonstrated *in vitro*, by exposing lymphocytes to enzymatic systems that generate O_2-derived products (Sagone *et al.*, 1978; Hoffeld *et al.*, 1981), to normobaric O_2 (Karlberg *et al.*, 1981; Kraus *et al.*, 1985) or to different oxidizing reagents (Chaplin & Wedner, 1978; Fishman *et al.*, 1981; Noelle & Lawrence, 1981b). In particular, reagents like diamide and 2-cyclohexene-one have been demonstrated to decrease [^{3}H]TdR incorporation by lectin-stimulated human lymphocytes. Because these drugs were maximally effective when added during the first 6 hr of stimulation, it has been suggested that they inhibit a very early event in the cell cycle. Our finding that O_2 exposure blocked this cycle early, before the G1 phase, is in accordance with this suggestion.

Inhibition of mitogen-induced lymphocyte proliferation by oxidative agents is thought to be related to alterations in membrane thiol groups and reduced Glutathione (GSH) metabolic pathways (Fishman *et al.*, 1981; Noelle & Lawrence, 1981a, b). The thiol/disulphide status of the cell is indeed of crucial importance for cellular homeostasis since, in particular, it affects the synthesis of certain macromolecules, including proteins, RNA and DNA (Kosower & Kosower, 1978). Further, Ohmori & Yamamoto (1982, 1983) have shown that addition of 2-ME to the culture medium enhances proliferation by facilitating cellular uptake of L-cystine, through the formation of a mixed disulphide cys-2-ME which is readily metabolized intracellularly into L-cysteine and glutathione. In addition, 2-ME has been shown to protect rat and mouse cells against *in vivo* or *in vitro* oxidative injury (Kraus *et al.*, 1985; Gougerot-Pocidalo *et al.*, 1985), by maintaining both the proliferative response and cell sulphhydryl content. Consequently, we attempted here to analyse the effects of the thiol compound 2-ME on the cellular events that were modified when human PHA-stimulated PBMC were exposed to O_2. We show that, like mouse and rat lymphoid cells, human PBMC can be protected against oxidative injury by 2-ME. Thus, in PBMC, the percentage of the proliferative response to PHA, expressed as the ratio of O_2 c.p.m./air c.p.m., increased from $41 \pm 5\%$ without 2-ME, to $80\% \pm 5\%$ with 2-ME (Table 1). Further, 2-ME almost protected PBMC against the O_2-induced cell cycle blockade in G0 phase, allowing the cell cycle to progress better from the G0 to S phase. The reduction by O_2 of the expression of IL-2 and transferrin receptors known to appear in the G1 phase may be related to the decreased percentage of cells progressing from G0 to G1. In addition, the expression of these receptors was protected by 2-ME (Table 4), as was the cell cycle progression. These protective effects cannot be due to increased cell viability, because the numbers of viable cells were not significantly different when the cultures under normobaric oxygen were incubated with or without 2-ME (Table 2). Note that neither O_2 exposure nor 2-ME changed the lymphoid CD3, CD4 or CD8 subset distribution of PBMC (data not shown).

Consequently our results show that oxidative injury exerts its effects in the early phases of the cell cycle, and that thiol compounds protect these early steps from the oxidative injury.

The reduced number of cells entering the early phase of the cell cycle may be due to impaired PHA binding to the cell membrane. However, it has been shown previously that exposure to O_2 does not modify mitogen binding to this membrane (Gougerot-Pocidalo *et al.*, 1985). Moreover, as discussed below, the increased IL-2 production by PHA-stimulated PBMC observed here under exposure to O_2 suggests that PHA binding to these cells did not alter. Our results indeed show that IL-2 activity increases in the supernatants of PHA-stimulated PBMC exposed to O_2 (Fig. 2). Quantification of IL-2 supernatant activity must take into account that this activity is produced in a culture system containing both 'producer' cells and cells that absorb and consume IL-2 via IL-2 receptors (Stadler *et al.*, 1981). The fact that normobaric O_2 exposure reduced mitogenic proliferation partly explained the IL-2 accumulation in the supernatants of cells exposed to O_2. However, after pretreatment with mitomycin *c*, IL-2 activity was always enhanced in the supernatants of exposed cells versus control cells (Fig. 2a). This finding may be due partly to the reduced IL-2 receptor expression of cells exposed to O_2. However, 2-ME, which protected IL-2 receptor expression (Table 4), did not modify the IL-2 activity recovered in the supernatants of cells pretreated

with mitomycin *c* and exposed to O_2 (Fig. 2b). Therefore, it may be suggested that O_2 exposure enhances IL-2 release, but depresses proliferation. Bettens *et al.* (1982) have shown that IL-2 activities could be recovered 6–10 hr after PHA stimulation, and therefore that IL-2 production was initiated before the major G1a cell formation took place. Thereafter, IL-2 titres increased and peaked in G1. Therefore, it may be inferred that in the present work, IL-2 production was increased by O_2 exposure, either because the number of cells not yet in G1 increased and/or because the rate of IL-2 release by these cells or by the cells that 'escaped' to G1 was increased.

Roth & Dröge (1987) have shown that H_2O_2 modulated IL-2 production and [^{3}H]TdR incorporation of macrophage-depleted spleen cells in the same way. However, in our study, we did not deplete the cells of monocytes. Because the role of IL-1 in the production of IL-2 by PHA-stimulated human PBMC is known (Smith *et al.*, 1980; Williams *et al.*, 1984), we investigated the effect of exposure to O_2 on IL-1 production by PHA-stimulated human monocytes. Our results demonstrate that this exposure increased both extracellular and cell-associated IL-1 activity, thus providing evidence that total IL-1 production by the monocytes exposed to O_2 was enhanced. The enhanced IL-2 activity recovered in the supernatants of PHA-stimulated PBMC exposed to O_2 therefore may be due to the decrease in the proliferation and IL-2 receptor expression of the T lymphocytes, and to the rise in IL-2 production by these lymphocytes in response to the enhancement of IL-1 production by the monocytes. This enhancement was not modified when 2-ME was added to the culture medium, and is currently under investigation.

In conclusion, the approach used here suggests that oxidative injury induced by exposure to normobaric O_2 reduces the proliferation of human PBMC by interfering with the cellular events that lead to the transition from the G0 to the G1 phase of the cell cycle. The protective effects of 2-ME suggest that thiol compounds have a critical role in the early events of this cycle. By contrast, exposure to O_2 induces increases in the production of both IL-1 and IL-2 that may not be related to alterations in the thiol status of the cell.

ACKNOWLEDGMENTS

We are grateful to Service Commun INSERM-PASTEUR trieur de cellules for cytofluorographic analyses and to B. Boitte for typing the manuscript.

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