Characterization of immunological depression in mice exposed to normobaric oxygen

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SUMMARY

Immunological cell functions were evaluated during 24, 48 and 96 h O_2 exposure in C57Bl/6 mice. A normobaric O_2 exposure resulted in depression of delayed type hypersensitivity (DTH) to oxazolone and *Staphylococcus aureus* antigens. This effect was proportional to the duration of O_2 exposure. The antibody response of splenic cells was more rapidly (24 h O_2 exposure) and markedly depressed using a T-dependent antigen (sheep red blood cell, SRBC) than with a T-independent antigen (trinitrophenylated lipopolysaccharide, TNP-LPS). While mitogen-induced proliferative responses of spleen cells to Con A and PHA were inhibited after 72 h of O_2 exposure, proliferative responses to LPS were inhibited after 96 h. A dissociated antigen and mitogen responses was observed after a short time of O_2 exposure (48 h): the antigen specific responses were impaired with a more pronounced effect on T lymphocytes, whereas the DNA synthesis in response to mitogen remained normal.

Keywords oxygen immunodepression delayed type hypersensitivity primary antibody response mitogen response

INTRODUCTION

Normobaric oxygen therapy is often employed in patients with severe respiratory illnesses to avoid the consequence of tissue hypoxemia. Yet prolonged exposure to 1 atmosphere (ATA) carries a risk of tissue damage. Lungs are exposed directly and are the most severely damaged in animal models (Clark & Lambertsen, 1971).

The symptoms are those of progressive respiratory distress until death. Despite many investigations, the mechanisms of O_2 damage remain unresolved. Direct O_2 toxicity is one possible explanation (Frank & Massaro, 1980). Another proposed hypothesis was an increased susceptibility to pulmonary infection, as suggested by studies on lung bacterial clearance (McCarthy *et al.*, 1972; Shurin, Permutt & Rilley, 1971). Moreover it has been proposed that O_2 pulmonary alveolar macrophages phagocytic activities after *in vivo* exposure were impaired (Rister, 1982; Jacquet & Gougerot-Pocidalo, 1983), increased (Murphey *et al.*, 1975) or unaltered (Fisher, Diamond & Mellen, 1974). This discrepancy led to the investigation of the involvement of the immune system in O_2 toxicity in mice (Gougerot-Pocidalo, Jacquet & Pocidalo, 1982) and in rats (Rouveix *et al.*, 1982). It has been found that 100% normobaric O_2 depressed the lymphoid tissue with a marked decrease in weight of thymus and spleen and their cell numbers after 48 h O_2 exposure. This lymphoid involution was found not to be mediated by modification of adrenal steroid activity (Gougerot-Pocidalo *et al.*, 1983).

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In this study we report on the effects of exposure to O_2 at various time intervals on some of the immune functions in mice.

MATERIALS AND METHODS

Animals. Female C57Bl/6 mice were used throughout the study (CSEAL, Orléans-La Source, France). The animals were 6–8 weeks old and were randomly selected for experimental and control studies.

O_2 exposure conditions. The exposure chamber used has been previously described (Charbonneau *et al.*, 1982). The O_2 concentration (95·5–100% O_2 at a pressure of 1 ATA) was continuously measured with a paramagnetic analyser, and varied by less than 1%. The ambient temperature was 22–23°C. Animals were allowed food and water *ad libitum* during the exposure period.

Preparation of cell suspensions. The spleen were removed from the donor animals, finely minced with scissors and gently blended with loose fitting glass grinder in Eagle's minimum essential medium (MEM). Cell suspensions were filtered through a cotton gauze sponge, washed twice in MEM and centrifuged at 300*g* for 5 min. Cell viability (trypan blue exclusion) was found to be greater than 90%.

Delayed type hypersensitivity (DTH) reactions

Antigen preparation. The antigen used were oxazolone (BDH Chemicals Ltd, England) and *Staphylococcus aureus* ATCC 25923 (obtained from the Microbiology Department, Hôpital Claude Bernard, Paris, France). The latter were grown in trypticase soy broth. Bacterial cells were collected by centrifugation and washed three times in phosphate-buffered saline (PBS) 0·15 M, pH 7·2. The cells were then resuspended in PBS and disrupted by sonication (Bransove Sonic Powel, USA) until no intact cell could be seen using microscopic checking. Following centrifugation at 10,000*g* for 45 min, the supernatant was collected and used as antigen. This was passed through a 0·22 μm Millipore filter before use. Protein concentration was determined by Lowry's method.

Sensitization. Mice were sensitized either by oxazolone or by *S. aureus*.

Oxazolone was administered percutaneously on the abdomen of previously shaved mice (0·1 ml of a 3% concentration in acetone). Other mice were sensitized by weekly subcutaneous injections of 10^8 CFU of viable *S. aureus*, in PBS (0·2 ml). Control animals received acetone alone or 10^8 of heat killed (100°C for 10 min) *S. aureus* in PBS.

O_2 exposure. Oxazolone sensitized mice were exposed to pure normobaric oxygen for 24, 48 and 72 h respectively 7 days after sensitization. *S. aureus* sensitized mice were exposed at the end of the third week of sensitization for a similar length of time.

Measurement of delayed type hypersensitivity (DTH). At the end of exposure, oxazolone sensitized mice were tested for DTH by an ear swelling assay: 20 μl of the antigen solution (2%) was administered to the skin of both ears. The increase in ear swelling was recorded using a skin calipers (Schnelltäster, Koplin, AO 2T). The results were expressed as the increase in ear thickness obtained by substracting the average thickness of both from that measured before antigenic challenge.

Similarly, other mice were tested for DTH to *S. aureus* by a footpad assay. The mice were injected in the left hind footpad with 0·025 ml of antigen in PBS and in the right hind footpad with the same volume of PBS as a control. The increase in footpad swelling was measured with the skin calipers at 0, 4, 24 and 48 h. Net foodpad swelling was determined by calculating the difference in swelling between both hind footpads. Thickness are expressed in units of 10^{-3}m.

Primary antibody response

Antigen preparation. The T-dependent antigen used was sheep red blood cells (SRBC) (Pasteur Institut, France). This was washed three times in NaCl, 0·9% and resuspended at a concentration of 5×10^8 erythrocytes/ml.

The T-independent antigen, trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared according to the method of Fidler (1975) and had a concentration of 500 μg/ml in NaCl, 0·9%.

Sensitization. Mice were immunized with one i.p. injection of 0·2 ml (1×10^8 erythrocytes/ animal) of a washed SRBC suspension (5×10^8 erythrocytes/ml) or TNP-LPS solution (100 μg/animal).

O_2 *exposure.* Mice were exposed to oxygen for 24, 48 or 72 h. In order to detect the best time of immunization in connection with the exposure period, various initial assays were performed. Antigen was then injected at different times before or after O_2 exposure. As no differences were observed between the above preliminary assays, it was decided to do the antigen injection immediately prior to O_2 exposure.

Direct haemolytic plaque assay. Mice were killed on day 4 and 5 after TNP-LPS and SRBC injection respectively. A modification of the technique of Cunningham & Szenberg (1968) was used to determine the number of splenic plaque forming cells (PFC).

Antibody forming cells were determined either by adding SRBC or TNP previously linked to SRBC (Fidler, 1975). The complement used in the assay was guinea pig serum which was twice absorbed with SRBC at 4°C for 20 min.

Mitogen stimulation of splenic cells

Mice were exposed to O_2 for 24, 48, 72 or 96 h. At the end of the time exposure, the animals were killed. Splenic cells were suspended in RPMI 1640 medium (GIBCO) supplemented with 25 mM HEPES, 2 mM glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin, 100 mM pyruvate, 1% non-essential amino acids (GIBCO) and 5% heat-inactivated fetal calf serum. Cultures were set up in microtest plates (Falcon Microtest II). Six hundred thousand cells in 0·2 ml culture medium were incubated in triplicate in the presence of previously determined optimal concentrations of mitogens: 2 μg/ml concanavalin A (Con A, Miles Yeda) or 20 μg/ml phytohemagglutinin (PHA, DIFCO) or 25 μg lipopolysaccharide (LPS from *E. Coli* 0·55: B5). Cultures were incubated at 37°C in 5% CO_2 enriched and humidified atmosphere for 48 h (LPS) or 72 h (Con A, PHA). One microcurie of ^{3}H-thymidine (specific activity 2 Ci/mmol) (CEA, Saclay, France) was introduced 20 h before the end of the culture. Cells were aspirated onto glass fibre strips by means of a Skatron cell culture harvester. Uptake of ^{3}H-thymidine was determined by counting in a liquid scintillation spectrophotometer (Intertechnique). Results were expressed as Δ ct/min (ct/min in mitogen stimulated cells − ct/min in unstimulated controls) ± s.e.

RESULTS
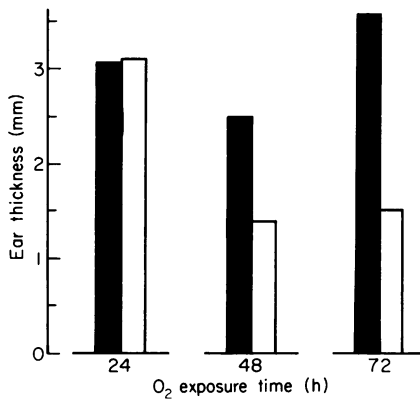
Delayed hypersensitivity

Ear reactions to oxazolone antigen were measured after 0, 24 and 72 h O_2 exposure. The 24 h exposed mice responded as the non-exposed group, whereas following 48 and especially 72 h O_2 exposure, the ear thickness decreased significantly (Fig. 1) to as low as 50% of the non-exposed control mice (Fig. 2).

A typical pattern of footpad reaction observed when the mice were given three injections of 10^8 CFU viable *S. aureus* and challenged with 100 μg of *S. aureus* is shown in Fig. 3. Strong footpad reactions were seen from 4 to 24 h. These reactions became weaker and weaker after O_2 exposure. The peak of swelling declined to a minimum when the mice were exposed to pure normobaric oxygen during 48 h (Fig. 3).

Primary antibody response

In vivo *response to SRBC immunization.* Animals immunized with T-dependent SRBC prior to O_2 exposure were tested for their PFC responses 5 days later. Table 1 shows a reduction in the PFC response compared to normal mice. This decrease reached a maximum after 48 and 72 h of O_2 exposure ($P < 0.001$).

In vivo *response to TNP-LPS immunization.* The number of PFC responses was similarly reduced after 48 and 72 h of O_2 exposure ($P < 0.001$). It is interesting to note that, unlike the SRBC PFC responses, there is no significant decrease after 24 h O_2 exposure (Table 2).

Fig. 1. Net ear swelling of mice following percutaneous administration of oxazolone. ■ = control mice ($n = 6$); □ = oxygen treated mice ($n = 6$).
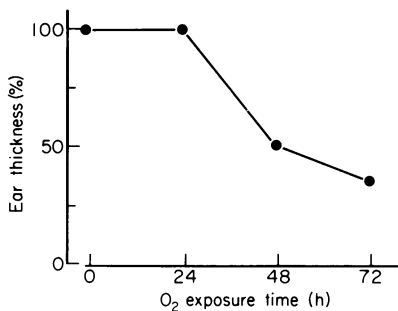
Fig. 2. Ear response of mice to oxazolone delayed hypersensitivity: effect of various time of O_2 exposure on the response.
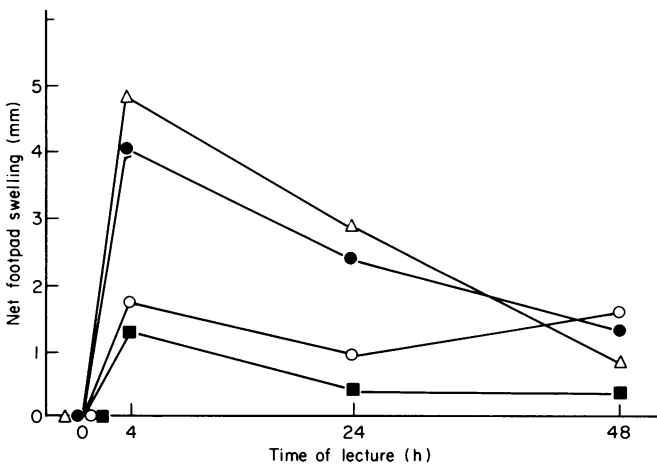
Fig. 3. Net footpad swelling of mice following three injections of 10^8 CFU of viable *S. aureus*. All the mice were challenged with 100 μg *S. aureus* antigen. △ = control animal ($n = 8$); ● = 24 h O_2 exposure ($n = 6$); ○ = 48 h O_2 exposure ($n = 6$); ■ = 72 h O_2 exposure ($n = 6$).

Table 1. Plaque forming cells (PFC) response to SRBC was studied 5 days after *in vivo* immunization

	Control ($n=18$)	O_2 exposure time (h)		
		24 ($n=8$)	48 ($n=10$)	72 ($n=8$)
Number of cells per spleen ($\times 10^6$)	152.8 ± 14.7	117 ± 13.5 (NS)	96.6 ± 7.5 ($P<0.02$)	38.5 ± 6.6 ($P<0.001$)
PFC per spleen	44,000	16,000 ($P<0.01$)	129 ($P<0.001$)	29.4 ($P<0.001$)
PFC per 10^6 cells	302.2 ± 35	154.8 ± 36 ($P<0.05$)	1.6 ± 0.5 ($P<0.001$)	1.4 ± 0.8 ($P<0.001$)

    C57Bl/6 mice were exposed to pure normobaric O_2 immediately after immunization.
    Results are expressed as the mean value $\pm$ s.e.
    Each O_2 exposed group was compared to the control by the Student's *t*-test.

Table 2. Plaque forming cells (PFC) response to TNP-LPS, was studied 4 days after *in vivo* immunization

	Control ($n=19$)	O_2 exposure time (h)		
		24 ($n=17$)	48 ($n=18$)	72 ($n=10$)
Number of cells per spleen ($\times 10^6$)	95.8 ± 9	84.3 ± 8 (NS)	70.6 ± 5 ($P<0.02$)	45.7 ± 5 ($P<0.001$)
PFC per spleen	15,110	13,600 (NS)	3,090 ($P<0.001$)	2,730 ($P<0.001$)
PFC per 10^6 cells	183.1 ± 20	169.7 ± 16.4 (NS)	47.6 ± 7 ($P<0.001$)	64.9 ± 9 ($P<0.001$)

    C57Bl/6 mice were exposed to pure normobaric O_2 immediately after immunization.
    Results are expressed as the mean value $\pm$ s.e.
    Each O_2 exposed group was compared to the control by the Student's *t*-test.

Response to mitogens

The incorporation of ^{3}H-thymidine by splenic cells in response to mitogen is shown in Table 3. There was an obvious difference between the response to both Con A and PHA as compared to LPS. The former were significantly decreased after 72 h O_2 exposure whereas the latter were only diminished after 96 h which is the time of dramatic depression of immune cells.

DISCUSSION

The effect of pure normobaric inhaled oxygen on immune cells *in vivo* has been recently studied. This induced an involution of the lymphoid system with a decrease in thymic and splenic weights and cell counts depending upon the length of O_2 exposure. These facts have been demonstrated in mice (Gougerot-Pocidalo *et al.*, 1982) and in rat (Rouveix *et al.*, 1982). In this study, specific immune function assessed either by DTH or primary direct PFC responses are suppressed as are

Table 3. O_2-dependent modification of spleen cell responsiveness to PHA, Con A and LPS stimulation in C57Bl/6 mice

O_2 exposure time (h)	Mitogen	Control	O_2 exposure
48 (*n*=4)	Con A	161 ± 14	82 ± 15
	PHA	26 ± 7	32 ± 4
	LPS	38 ± 6	39 ± 7
72 (*n*=4)	Con A	153 ± 12	49 ± 6
	PHA	28 ± 8	20 ± 3
	LPS	31 ± 6	25 ± 2
96 (*n*=4)	Con A	189 ± 15	29 ± 4
	PHA	32 ± 6	15 ± 3
	LPS	48 ± 8	18 ± 4

Results are expressed as Δct/min $\times 10^3$ (ct/min. in mitogen stimulated cells $-$ ct/min in controls) $\pm$ s.e.
The mean values of the four different experiments are with pooled spleen cells.

spleen cell responses to mitogens. The magnitude of these effects is correlated to the time of O_2 exposure. Moreover there are several difference according to the type of immune response.

After 24 h of hyperoxia, the only modification is a significant decrease ($P < 0.05$) of the PFC responses to SRBC which is a T-dependent antigen. On the contrary the PFC responses to TNP-LPS, a T-independent antigen, are conserved. Similarly, DTH responses do not vary. After one more day of exposure (total = 48 h), one can see a significant decrease of the TNP-LPS responses ($P < 0.001$) whereas those of SRBC antigen are dramatically reduced. At the same time, the DTH responses only start to diminish.

These observations are in contrast with the maintenance of the lymphoid organ cellularity which is only depleted after 72 h O_2 exposure (Gougerot-Pocidalo *et al.*, 1982). Moreover, mitogen-induced proliferative responses of splenic cells are still normal at 48 h, despite the fact that one can observe a weak decrease using Con A stimulation. The markedly O_2 inhibitory effect on mitogen-induced lymphoproliferative responses of splenic cells is maximal at 72 h for T cell mitogens and at 96 h for the B cell mitogen LPS. This latter time corresponds to the pre-lethal onset of O_2 toxicity.

These results strongly suggest that O_2 injury to immune cells is associated with impairment of specific immune responses, and that T lymphocytes rather than B lymphocytes are the more sensitive. The suppressive effect on DNA synthesis only appears at a later stage. It is interesting to note that, in this work, short-term exposure to normobaric O_2 (48 h) does not trigger a definitive suppression of antibody response as the PFC to SRBC antigen were found normal 10 days after recovering from the exposure time.

It is important to note that the modifications of the immune response precede the development of the pathological changes in the lung. Indeed, O_2 lung damage (exudative phase with alveolar edema) occur within 48–72 h of exposure in mice (data to be published). Moreover it is known that no hypoxemia occurred despite the pulmonary injuries. Pa_{O_2} only fell at a pre-terminal state (Charbonneau *et al.*, 1982).

The basic mechanisms underlying the O_2 depressed immunological responses remain unknown. It has been previously proposed that *in vivo* hyperoxia could induced O_2 derived free radicals (McCord & Fridovich, 1978). On the other hand, *in vitro* increase in O_2- derived free radicals can lead to lymphocyte damage as demonstrated by the work of many laboratories. It has been shown that the addition of oxidase enzymes such as xanthine/xanthine oxidase to cultures inhibits PHA stimulated human peripheral blood lymphocytes (Sagone, Kamps & Campbell, 1978) and Con A

stimulated murine spleen cells (Hoffeld, Metzger & Oppenheim, 1982). In addition, these authors gave good evidence to show that the *in vitro* primary antibody response was negatively influenced by oxygen radicals and that this could be reversed by oxygen scavengers. Grever *et al.* (1980) assessed that the lymphocyte capacity to kill non-antibody and antibody coated tumour cells was also impaired. Finally, numerous studies have demonstrated that various oxygen radicals may interact with DNA (Morgan, Cone & Elgert, 1976).

It is of interest to note that other oxidants can induced lung damage and altered immune functions such as ionizing radiation (Gross, 1977; Doria, Agarossi & Adorini, 1982) or nitrogen dioxide (Mustafa & Tierney, 1978; Fujimaki, Shimizu & Kubota, 1981) and that this could be mediated through O_2 derived free radicals (Gershman *et al.*, 1954).

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