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ORIGINAL ARTICLE

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Effect of hypobaric hypoxia on immune function in albino rats

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Abstract The effect of exposure to hypoxia on macrophage activity, lymphocyte function and oxidative stress was investigated. Hypoxia enhanced peritoneal macrophage activity as revealed by enhanced phagocytosis and free radical production. There was no significant change in antibody titres to sheep red blood cells in either serum or spleen during hypoxia. However, there was a considerable reduction in the delayed-type hypersensitivity response to sheep red blood cells, indicating the impairment of T-cell activity. Hypoxia decreased the blood glutathione (reduced) level and increased plasma malondialdehyde by a factor of about 2. It is therefore speculated that hypoxia imposes an oxidative stress leading to decreased T-cell acivity.

Key words Hypoxia · Macrophage activation · Oxidative stress · Immunity

Introduction

The effect of chronic hypoxia on immunity is controversial. There are contrasting reports on the effect of hypobaric hypoxia on immune functions. Early work showed that exposure to hypoxia resulted in increased immunoglobulin levels with concomitant resistance to viral infection (Trapani 1966). Chohan et al. (1975) reported increased serum IgG and IgA levels both in high-altitude natives and in sea-level natives inducted to high altitudes, compared to sea-level residents. In contrast, Mirrakhimov and Kitaev (1979) and Kitaev and Tokhtabayev (1981) reported increased infant mortality to respiratory infections among high-altitude natives. Meehan (1987) reported that hypoxic exposure made experimental animals more susceptible to bacterial infections. The latter observations suggest that the host defence mechanisms might be impaired in hypobaric hypoxia.

Although some work has been carried out on the effect of hypoxia on humoral immunity, little work has been done on cell-mediated immunity especially on the response of macrophages. Further, the factors regulating immunomodulation in hypoxia are not clear at present. The involvement of free radicals in immunomodulation is of considerable interest as it is well established that hypoxia imposes an oxidative stress (Edmonds and Blake 1994). The cells of the immune system are particularly sensitive to changes in oxidant stress because of the high content of polyunsaturated fatty acids in their plasma membranes (Coquette et al. 1986; Meydani et al. 1995). Further, these cells are often exposed to reactive oxygen intermediates produced as a normal part of their function. Oxygen free radicals (ORFs) such as superoxides, hydroxyl ions and nitrite radicals, generated by activated macrophages for defence mechanisms, may also act as mediators of inflammation if produced in excess (Joe and Lokesh 1994).

In view of the above, the present study was carried out to find the effect of hypoxia on some aspects of immune function such as macrophage activity, the humoral response and the delayed-type hypersensitivity (DTH) response. Further, an attempt has been made to explain the possible mechanisms of immunomodulation in hypoxia.

Methods

Animals

Animals from a locally bred colony of male Sprague Dawley albino rats maintained under the principles of laboratory care were used. They weighed approximately 200 g and were reared in our laboratory. The animals were maintained at 25±2° C and fed with a standard pellet diet and water ad libitum with light and dark cycles of approximately 12 h each.

Hypoxia exposure

The rats were exposed to a simulated altitude of 7576 m (25000 ft) in a decompression chamber for varying periods – 6 h daily for ei-

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ther 7 or 15 days. To determine the effect of acute, short-term hypoxia on macrophage activity, the animals were exposed to hypoxia for 2 h. The temperature inside the chamber was maintained at 32±2° C (to prevent the effect of hypothermia) and the flow rate of air was maintained at 4 l min^{-1}. After hypoxic exposure, the animals were allowed to recover for 1 h at room temperature (30° C) before the analyses were carried out.

Sheep red blood cells

The sheep red blood cells (SRBC) were collected in Alsever's solution, washed three times with pyrogen-free saline and adjusted to 5×10^9 cells ml^{-1} for immunization and challenge.

Total leukocyte counts/differential leukocyte counts

After exposure to hypoxia, the blood was collected by retro-orbital puncture. Total leukocyte counts (TLC) were determined using a haemocytometer and a microscope . Differential leukocyte counts (DLC) were determined after carrying out Leishman's staining of blood smears.

Macrophage activation

Phagocytosis

Phagocytosis was determined by the method of Wang et al. (1991) with minor modifications. Briefly, after hypoxic exposure, the rats were taken out of the hypoxic chamber and 2 ml SRBC (10^{10} cells ml^{-1}) was injected intraperitoneally. After 1 h, the rats were sacrificed by cervical dislocation and 15 ml cold Hank's balanced salt solution (HBSS) was injected into the abdomen. After a brief abdominal massage, the peritoneal fluid was collected and washed three times with fresh HBSS. Finally, the cells were suspended in 0.5 ml HBSS, the smears were made and Leishman's staining was carried out. The macrophages showing phagocytosis of 1–5 SRBC were graded by counting at least 100 cells/slide. The percentage of phagocytosis and the Phagocytic Index (PI) were determined as follows:

$$\% \text{ phagocytosis} = \frac{\text{Number of macrophages having phagocytized SRBC}}{100 \text{ macrophages}}$$

$$\text{P.I.} = \frac{\text{Total no. of SRBC phagocytized}}{100 \text{ macrophages}}.$$

OFR generation (nitroblue tetrazolium reduction)

After suspending the peritoneal cells in HBSS, 100 µl of cells was plated into 96 well tissue culture plates and incubated for 2 h at 37° C in a CO_2 incubator for macrophages, to adsorb to the bottom of the plates. The cells were then washed three times with cold HBSS to remove non-adherent cells. Later, 200 µl of Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum was added, followed by the addition of 25 µl nitroblue tetrazolium chloride (NBT, 10 mg ml^{-1}). The cells were then incubated in a CO_2 incubator at 37° C for 1 h. Later, the crystals of NBT reduced were solubilized in 200 µl DMSO and 25 µl Sorenson's buffer, pH 10.5. The blue colour obtained was measured at 600 nm using an ELISA reader.

Humoral immune response

Serum antibody titre

Separate groups each of eight rats were immunized by injecting 20 µl of 5×10^9 SRBC ml^{-1} sub-cutaneously (s.c.) into the right hind food pad. The rats were exposed to hypoxia for 6 h daily for 7 days. After the last exposure, the rats were allowed to recover under normoxia for 1 h. Later, blood samples were collected from individual rats by retro-orbital puncture for antibody titre. Antibody levels were determined by haemagglutination as described earlier (Nelson and Mildenhall 1967). Briefly, equal volumes of individual serum samples of distinct groups were pooled. To serial 2-fold dilutions of pooled serum samples, made in normal saline containing 0.1% bovine serum albumin in V-bottomed Takasty microtitration plates, 25 µl of 0.1% SRBC was added. After mixing, the erythrocytes were allowed to settle down at 37° C until the control wells showed a negative pattern (small button). The value of the highest serum dilution causing visible haemagglutination was taken as antibody titre.

Splenic antibody titre

This was determined as described by Atal et al. (1986). Briefly, after hypoxic exposure and recovery, the rats were sacrificed by cervical dislocation and spleen cells were isolated by mincing spleen in cold HBSS. The cells obtained were washed three times with HBSS and finally suspended in HBSS and adjusted to a concentration of 2×10^6 cells ml^{-1}. To 1 ml spleen cells, 1 ml of 2% SRBC was added, followed by the addition of 1 ml of 10% guinea pig serum. After incubating the cells for 45 min at 37° C, the cells were pelleted down by centrifugation at 1500 rpm for 15 min. The colour of the supernatant was determined at 450 nm using a spectrophotometer.

DTH response

This was induced by the method of Atal et al. (1986). Groups of eight rats were immunised by injecting 25 µl of 1×10^{10} SRBC ml^{-1} s.c. into the right hind foot pad. After 7 days, the thickness of the left hind foot pad was measured using vernier calipers. The rats were then challenged by injecting 25 µl of 1×10^{10} SRBC ml^{-1} intradermally into the left hind food pad. Foot thickness was measured again 24 h and 48 h after the challenge. The difference between pre- and post-challenge foot thickness was taken as a measure of the DTH response.

Blood glutathione and plasma malondialdehyde levels

After hypoxic exposure and recovery, the animals were sacrificed and blood glutathione (GSH) was determined by the method of Kum-Talt and Tan (1974). Plasma malondialdehyde (MDA) levels were estimated as descirbed by Dousset et al. (1983).

All the experiments were performed on two different occasions on each of eight rats and the statistical analyses were carried out using a one-way ANOVA.

Results

TLC/DLC

The effect of hypoxia on TLCS and DLC (polymorphonuclear cells and lymphocytes) is shown in Table 1. There was no significant change in either TLC or DLC in the control animals or animals exposed to hypoxia.

Peritoneal macrophage activity

The effect of hypoxia on peritoneal macrophage activity, as determined by phagocytosis and NBT reduction, is shown in Figs. 1 and 2. The macrophage activity was en-

Table 1 Effect of hypoxia for 6 h daily for 7 days on total leukocyte count (*TLC*) and differential leukocyte counts (*DLC*). No significant difference was seen (*n*=8; values are mean±SE)

	TLC (cells/mm^3)	DLC (%)	
		Polymorphonuclear cells	Lymphocytes
Control	11683±1566	36±1.8	59±1.9
Hypoxia	10266±780	36±1.5	60±1.6

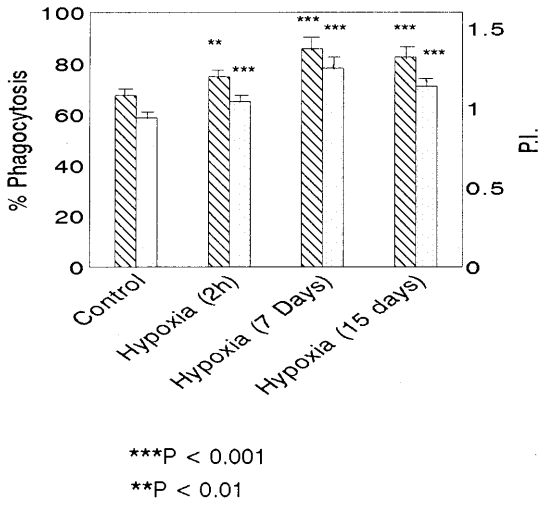

Fig. 1 Effect of hypoxia on phagocytosis of SRBC in peritoneal macrophages. The animals were exposed to hypoxia for either 2 h once, for 6 h daily for 7 days or for 6 h daily for 15 days. After hypoxic exposure, 2 ml SRBC was injected i.p. After 1 h, the rats were sacrificed by cervical dislocation and the macrophage activity was determined. (values are mean±SD). *PI* Phagocytic index. ▨ % Phagocytosis; ⊠ P.I.

hanced by hypoxia as evidenced by both % phagocytosis and PI (Fig. 1). With an increase in the duration of exposure to hypoxia (15 days), there seems to be no further increase in macrophage activity. The NBT reduction by peritoneal macrophages also showed a similar trend (Fig. 2).

Antibody response

The antibody titres in serum and spleen to SRBC challenge are shown in Table 2. There was a marginal fall in both serum and splenic antibody titres in the animals exposed to hypoxia compared with the control animals, but the difference is not significant.

DTH response

The DTH response to SRBC challenge decreased significantly in animals exposed to hypoxia for 7 days compared to the control animals. When hypoxic exposure was increased to 15 days, the DTH response decreased further (Table 3).

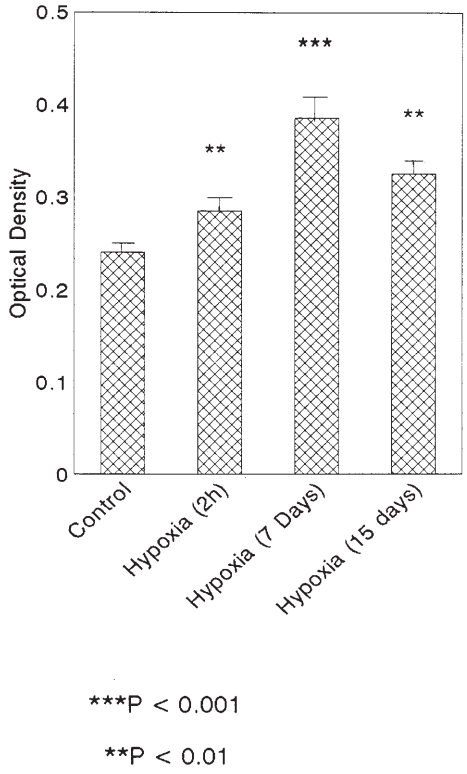

Fig. 2 Effect of hypoxia on NBT reduction in peritoneal macrophages. The animals were exposed to hypoxia for either 2 h once, for 6 h daily for 7 days or for 6 h daily for 15 days. The peritoneal macrophages were isolated and NBT reduction was measured (values are mean±SD)

Table 2 Effect of hypoxia for 6 h daily for 7 days on humoral immune respone (*n*=8; values are mean±SE). No significant difference was seen

	Antibody titres			
	Serum	% of control	Spleen	% of control
Control	84.33 ±0.02	–	0.333 ±0.02	–
Hypoxia	77.50 ±2.70	92.0	0.289 ±0.030	87.1

Table 3 Effect of hypoxia for 6 h daily on DTH response to SRBC (*n*=8; values are mean±SE)

	Control	Hypoxia (7 days)	Hypoxia (15 days)
Increase in foot pad thickness (mm)	0.70±0.05	0.46±0.06***	0.37±0.079***
% of control	–	65.71	52.86

*** $P<0.001$

GSH and MDA levels

The experiments conducted to investigate the effect of hypoxia on oxidative stress revealed a two-fold increase in plasma MDA levels in animals exposed to hypoxia

Table 4 Effect of hypoxic exposure for 6 h daily for 7 days on blood glutathione (reduced) and plasma malondialdehyde levels. (n=8; values are mean±SE)

	Blood glutathione (mg 100 ml^{-1})	Plasma malondialdehyde (nmol ml^{-1})
Control	43.38±1.30	2.407±0.068
Hypoxia	24.35***±1.46	5.175***±0.054

*** P<0.001

compared with the controls. In contrast a two-fold reduction in blood GSH levels was noticed in animals exposed to hypoxia compared to the controls (Table 4).

Discussion

The effect of hypobaric hypoxia on peritoneal macrophage activity is not well documented. The present study indicated that hypoxia activated phagocytosis of the peritoneal macrophages. NBT reduction is used to measure OFR production by phagocytes (Joe and Lokesh 1994). Our studies showed increased NBT reduction by peritoneal macrophages isolated from animals exposed to hypoxia compared with control animals. As there was no further increase in either phagocytosis or NBT reduction with increased duration of hypoxic exposure, it was evident that the effect was not cumulative. This observation is in agreement with the recent report that anoxia enhanced macrophage activity, resulting in enhanced cytokine release (Albina et al. 1995). The drop in macrophage activity on prolonged hypoxic exposure was primarily due to adaptation rather than cytotoxicity. This was confirmed by vital staining of peritoneal macrophages isolated from both control and hypoxic animals, by studying the uptake of trypan blue or neutral red (data not shown). It has been reported that OFRs are formed by a variety of activated cells in addition to macrophages, such as neutrophils, eosinophils and monocytes during stress (Babior and Woodman, 1990).

Unlike the macrophage activity, in the present study the antibody response to SRBC (T-dependent antigen) was, by and large, unaffected both in serum and in the spleen. Although there was a marginal fall in antibody titre in hypoxia-exposed animals, the effect was not significant. Earlier studies have shown that there is an elevation in serum antibody titres to the antigenic stimulus in animals exposed to hypoxic stress (Trapani 1966). In contrast, more recently Biselli et al. (1991) reported that there was no change in B-lymphocyte response against *Neisseriae meningitidis* PsA and PsC (T-independent) antigens and our results confirm this observation. However, we found that there was a significant drop in the DTH response which revealed that, during hypoxia, there is an impairment of T-lymphocyte activity (cell-mediated immunity). Earlier Meehan et al. (1988) also found a decreased T-cell proliferative response in humans exposed to hypoxia.

There is widespread evidence to indicate that hypoxia imposes an oxidative stress on cells (Borg 1993; Schnass 1994). To investigate whether the observed immunosuppression by hypoxia is due to free radical generation, we determined the plasma MDA and blood GSH levels. In the present study it was found that there was a two-fold increase in plasma MDA with a concurrent decrease in blood GSH levels in animals exposed to hypoxia compared with controls. This indicated that there was an imposition of oxidative stress during hypoxia.

Earlier studies have revealed that oxidative stress inhibits the T-cell response (Flescher et al. 1994; Meydani et al. 1995). This was found to be due to a decrease in the antioxidant potential of the body. Numerous studies have demonstrated the role of GSH in immune function. Intracellular GSH has been proven to be essential for lymphocyte activation and proliferation (Franklin et al. 1990). It is speculated that GSH scavenges and protects the immune cells from OFRs by detoxifying them. Further, it was shown that GSH inhibits the synthesis of Prostaglandin E_2 (PGE_2) and Leukotriene B_4 (LTB_4) while stimulating IL-2 production (Gills et al. 1978; Shapiro and Meydani 1993). Since there was a reduction in blood GSH levels in animals exposed to hypoxia compared to the control animals, it is speculated that the generation of more OFRs by macrophages may be responsible for the observed immunosuppression of T cells.

It is therefore concluded that exposure to hypoxia resulted in enhanced OFR production by the activated macrophages and possibly by other activated cells such as polymorphonuclear cells. This in turn inhibited the activity of T cells leading to a decreased DTH response. Activated macrophages secrete suppressive factors such as H_2O_2 and prostaglandin E_2 both of which have been shown to depress lymphocyte function (Metzger et al. 1980; Fisher and Bostick-Bruton 1982). Betz and Fox (1991) reported that prostaglandins inhibit the production of T-helper TH_1-dependent lymphokines (mediators of cell mediated immunity) but not TH_2-dependent lymphokines (mediators of humoral immunity). In the present study, it was found that hypoxia did not alter the antibody response to SRBC. Whether this could be due to the production of prostaglandin E_2 or various cytokines by activated macrophages is a subject for further research.

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