

## Suppressive effect of hyperbaric oxygenation on immune responses of normal and autoimmune mice

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(Accepted for publication 15 May 1991)

### SUMMARY

We studied the effect of hyperbaric oxygenation (HBO) on immune responses in normal and autoimmune mice. Mice were exposed to HBO in an animal chamber at a pressure of 252.5 kPa for 1 h and once a day for 5 days. The immunization of C3H/He mice with sheep erythrocytes induced marked anti-sheep erythrocyte antibody response on day 7. However, this response was markedly suppressed in HBO-treated mice. The suppression is dependent on the duration of HBO and it works on the early and the late stage of antibody responses. HBO suppresses the development of both sheep erythrocyte-specific B cells and helper T cells after the immunization. Then, we tried to expose autoimmune mice to HBO. Spontaneous immunoglobulin production of NZB and MRL/lpr spleen cells was also significantly suppressed by HBO. Furthermore, long term HBO exposure results in the suppression of the development of autoimmune symptoms such as proteinuria, facial erythema and lymphadenopathy in MRL/lpr mice. All these results suggest that HBO is applicable for the treatment of autoimmune diseases.

**Keywords** HBO lupus mouse autoantibody

### INTRODUCTION

Hyperbaric oxygenation (HBO) therapy is clinically applied for patients with local hypoxia such as skin ulcer, cerebral infarction and sudden deafness. It is widely accepted that the marked increase of dissolved oxygen in the blood and tissues results in the promotion of recovery from such diseases. Some investigators demonstrated that the exposure of experimental animals to HBO induced the suppression of several cell-mediated immune responses such as allograft rejection (Jacob, Thurning & Sacksteder, 1978) and delayed type hypersensitivity responses to tuberculin protein (Warren, Sacksteder & Jacob 1978a). It is also reported that the functions of leukocytes and macrophages are suppressed by *in vitro* hyperoxidation (Murphy, Hyams & Fisher, 1975; Babior, 1978). However, there has not been a full examination of the mechanism of the immunosuppressive effect of HBO. In a previous report (Saito *et al.*, 1989) we showed that HBO markedly reduced the level of serum IgG, anti-DNA antibody and immune complexes in patients with systemic lupus erythematosus. This evidence suggests that HBO may have some regulatory functions on immune responses. In this paper we studied the effect of HBO on immune responses using sheep erythrocyte-immunized mice as a model system. Furthermore,

we tried to expose autoimmune mice to HBO in order to evaluate the therapeutic application of HBO to autoimmune diseases.

### MATERIALS AND METHODS

#### *Animals*

C3H/He, NZB and MRL/lpr mice were purchased from Seiwa Experimental Animal Ltd. (Yoshitomi, Japan) and bred in our laboratory. Six-month old female C3H/He, 4-month old female NZB and 2-month old female MRL/lpr mice were used for experiments.

#### *Exposure to HBO*

Mice caged in groups of 10 were exposed to HBO in an animal chamber at the pressure of 252.5 kPa for 1 h once a day. After at least 5 min of flushing, the exhaust valve was closed and the flow from the commercial oxygen tank was adjusted to the rate of compression of 252.5 kPa. At the end of the treatment, the tank was decompressed slowly (50.5 kPa/min).

#### *Immunization of mice*

C3H/He mice were immunized with an i.p. injection of  $5 \times 10^8$  sheep erythrocytes. Anti-sheep erythrocyte antibody response was detected by a direct haemolytic plaque-forming cell (PFC) assay using spleen cells.

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#### Preparation of T cells and B cells

Spleen cell suspensions were prepared from mice by gently dispersing the tissue in HBSS (Nissui Seiyaku Co., Tokyo, Japan) and erythrocytes were lysed by treatment with  $\text{Tris-NH}_4\text{Cl}$ . The spleen cells suspended in RPMI 1640 medium (Nissui Seiyaku Co.) containing 5% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY) were applied on nylon wool column (Fenwal Laboratories, Morton Grove, IL). After incubation of cells on the column for 1 h at  $37^\circ\text{C}$ , non-adherent cells were eluted dropwise. The cell recovery from the column was about 10% of the original cells applied. The recovered cells were composed of more than 95%  $\text{Thy1}^+$  cells and did not respond to B cell mitogen such as lipopolysaccharide (LPS) from *Escherichia coli* and were used as purified T cells. Spleen cells were also treated with anti- $\text{Thy1.2}$  monoclonal antibody (Cedarlane Laboratories, Ottawa, Canada) and rabbit complement (Cedarlane Laboratories) at  $37^\circ\text{C}$  for 1 h. The cell recovery was about 40% of the original cell numbers treated. The recovered cells were composed of more than 90% immunoglobulin-positive cells and did not respond to T cell mitogen such as concanavalin A and were used as purified B cells (Yamashita & Hamaoka, 1979).

#### Secondary antibody production against sheep erythrocytes

Purified B cells ( $2.5 \times 10^5$ ) and T cells ( $2.5 \times 10^5$ ) from HBO-treated or untreated C3H/He mice primed with sheep erythrocytes were mixed in several combinations and cultured with  $1 \times 10^6$  sheep erythrocytes in RPMI 1640 medium (Nissui Seiyaku Co.) containing 10% FCS using microtitre culture plates (Falcon No. 3072, Falcon Plastics, Oxnard, CA) at  $37^\circ\text{C}$  for 5 days in 5%  $\text{CO}_2$  and 95% air. Anti-sheep erythrocyte antibody response was detected by PFC assay on day 5.

#### Assay of antibody production by spleen cells

Antibody-producing cells were detected by a direct haemolytic PFC assay on slide chambers with sheep erythrocytes. In measuring total immunoglobulin production of spleen cells from NZB and MRL/lpr mice, immunoglobulin-producing cells were detected by a reverse haemolytic PFC assay on slide chambers with protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) coated sheep erythrocytes (Pierce *et al.* 1971; Gronowicz, Coutinho & Melchers, 1976). Indirect PFC, facilitated by rabbit anti-mouse IgG serum, were detected. The results were expressed as the mean numbers and s.e.m. of  $\text{PFC}/5 \times 10^5$  spleen cells in triplicate cultures.

#### Evaluation of clinical effect of long term HBO exposure

Two-month-old female MRL/lpr mice were exposed to HBO once a day for 2 months in the conditions described above. Then the survival of mice, body weight and weight of bilateral inguinal lymph node were measured. Urine protein was measured semiquantitatively by means of Combistics (Sankyo Co., Tokyo, Japan). A grading of 0–4 was made on the basis of the protein concentration in the urine, the five categories being 0–30, 30–100, 100–300, 300–1000 and 1000 mg/dl, respectively. Urine of each mouse was measured for 3 days and mean scores were averaged in both control and HBO group. Anti-nuclear antibodies in the sera were detected by ELISA. The results were expressed as the optical density units at 405 nm of wavelength.

#### Statistical analysis

Statistical analysis of the difference between the control and the experimental group was performed using non-parametric Mann-Whitney *U* test.

## RESULTS

#### Effect of HBO on anti-sheep erythrocyte antibody response

C3H/He mice were immunized with sheep erythrocyte, exposed to HBO at 252.5 kPa for 1 h once a day and anti-sheep erythrocyte antibody response was detected by a direct haemolytic assay using spleen cells at several intervals after immunization. As shown in Fig. 1, anti-sheep erythrocyte PFC responses of control mice were increased to the level of  $400/1 \times 10^6$  spleen cells on day 7 and then gradually decreased. However, anti-sheep erythrocyte PFC responses of HBO-exposed mice were markedly reduced to  $150/1 \times 10^6$  spleen cells, although the time course of antibody response of HBO-treated mice was similar to that of HBO-untreated mice. The number of spleen cells recovered and the cell viability determined by a trypan blue dye exclusion test were not different between both HBO and control groups. This result suggests that HBO has suppressive activity on antigen-induced immune responses.

#### Kinetic study of the suppressive effect of HBO on the antibody response

Next, we studied the timing of HBO exposure on the anti-sheep erythrocyte PFC response. We exposed sheep-erythrocyte-immunized mice to HBO from day 0 to indicated day and sheep

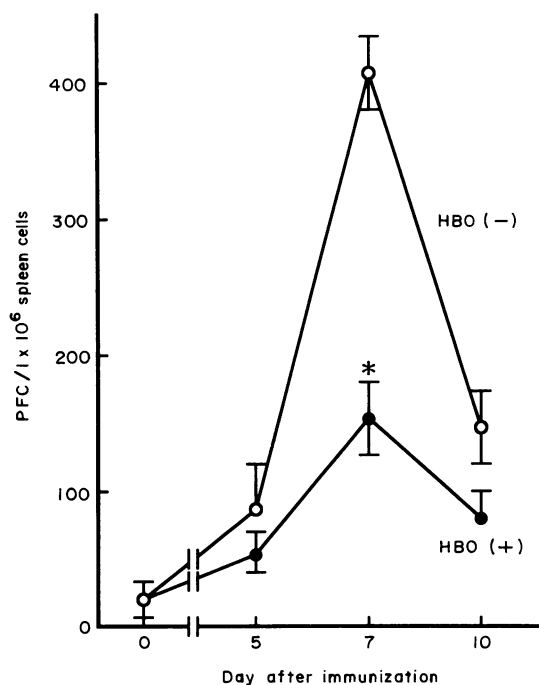
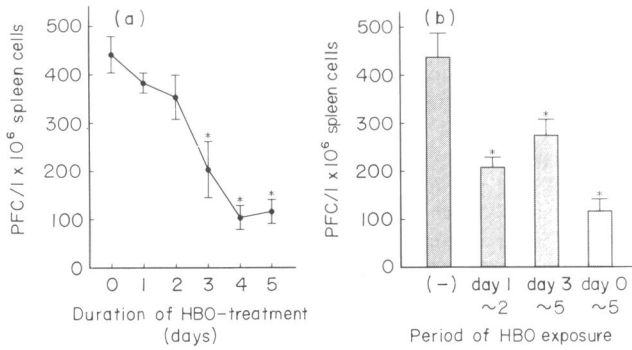
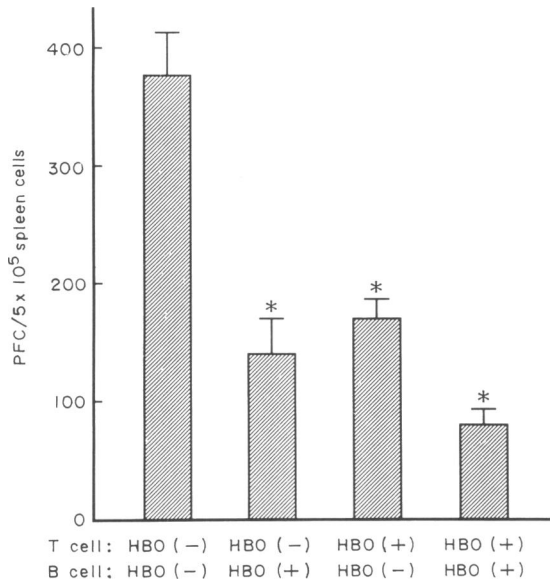


Fig. 1. Effect of HBO treatment on anti-sheep erythrocyte PFC response. C3H/He mice were immunized with  $5 \times 10^8$  sheep erythrocytes intraperitoneally on day 0. Then, the mice were exposed to HBO at 252.5 kPa for 1 h every day until killed. Anti-sheep erythrocyte PFC response was detected by a direct haemolytic plaque assay using spleen cells on indicated day. The results were expressed as the mean  $\text{PFC}/1 \times 10^6$  spleen cells  $\pm$  s.e.m. of five mice in each group. \* Significantly different from the control mice ( $P < 0.05$ ).

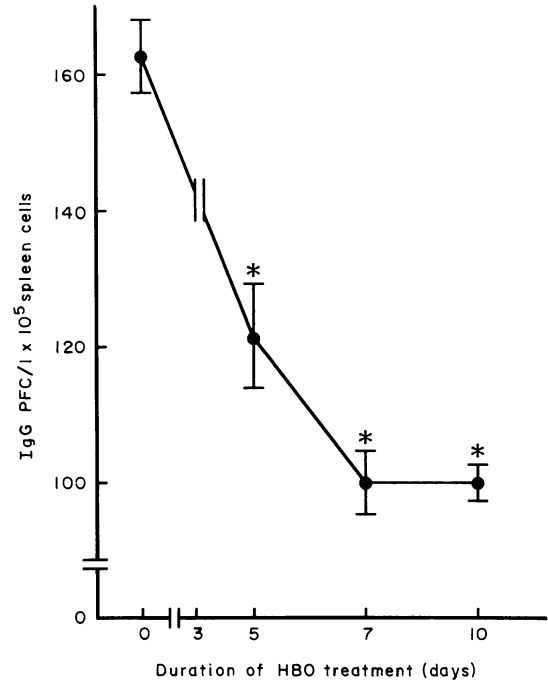


**Fig. 2.** Dose response of HBO on anti-sheep erythrocyte PFC response. (a) Sheep-erythrocyte-immunized C3H/He mice were exposed to HBO from day 0 to indicated day. (b) Sheep-erythrocyte-immunized C3H/He mice were exposed to HBO for indicated period. Anti-sheep erythrocyte PFC response of spleen cells was detected with a direct haemolytic assay on day 6. The results were expressed as the mean PFC/1  $\times$  10<sup>6</sup> spleen cells  $\pm$  s.e.m. of five mice in each group. \*Significantly different from the control mice ( $P < 0.05$ ).



**Fig. 3.** Effect of HBO treatment on B cell and T cell functions. Sheep-erythrocyte-immunized C3H/He mice were exposed to HBO for 7 days and then the spleen cells from each mouse were fractionated to B cells and T cells. T cells ( $2.5 \times 10^5$ ) and B cells ( $2.5 \times 10^5$ ) from both HBO and control groups were cultured together in combinations as indicated. After 5 days' culture with  $1 \times 10^6$  sheep erythrocytes, anti-sheep erythrocyte PFC response was detected with a direct haemolytic assay on day 5. The results were expressed as the mean PFC/5  $\times$  10<sup>5</sup> spleen cells  $\pm$  s.e.m. in triplicate cultures. \*Significantly different from the control mice ( $P < 0.05$ ).

erythrocyte PFC responses were detected on day 6. As shown in Fig. 2a, anti-sheep erythrocyte PFC response was already suppressed by one exposure and the suppression became more marked by the increase of the duration of exposure. Then, we studied which process through antigen recognition by lymphocytes to proliferation and differentiation into antibody-producing cells was suppressed by HBO. To do this we exposed sheep-erythrocyte-immunized mice to HBO at the early period or the



**Fig. 4.** Effect of HBO treatment on the spontaneous immunoglobulin production in NZB mice. Four-month-old female NZB mice were exposed to HBO for indicated days and the spontaneous immunoglobulin production of spleen cells was detected with a reverse haemolytic plaque assay. The results were expressed as the mean PFC/1  $\times$  10<sup>5</sup> spleen cells  $\pm$  s.e.m. of five mice in each group. \*Significantly different from the control mice ( $P < 0.05$ ).

later period after the immunization. As shown in Fig. 2b, a similar suppression of anti-sheep erythrocyte PFC response was observed in mice exposed for day 0–2 (early period) and day 3–5 (late period). However, the suppression is weaker than that of mice exposed for day 0–5 (total period). These results suggest that HBO does not suppress any specific process but generally suppresses the whole process and results in a reduction of the antibody response.

#### Effect of HBO on the function of T cells and B cells

Anti-sheep erythrocyte antibody production is induced by an interaction of B cells and helper T cells (Martin & Miller, 1968). The next question is, which cell type is suppressed by HBO treatment. To study this question we used an *in vitro* secondary stimulation system. We prepared purified T cells and B cells from spleens of HBO-treated and control mice which had been immunized with sheep erythrocytes. Then, T cells ( $2.5 \times 10^5$ ) and B cells ( $2.5 \times 10^5$ ) from each group were mixed and cultured for 5 days in the presence of  $1 \times 10^6$  sheep erythrocytes. As shown in Fig. 3, the anti-sheep erythrocyte PFC response was markedly suppressed when either B cells or T cells obtained from HBO-treated mice were cultured with B cells and T cells from control mice. These results suggest that HBO gives its suppressive effect on both T and B cell functions.

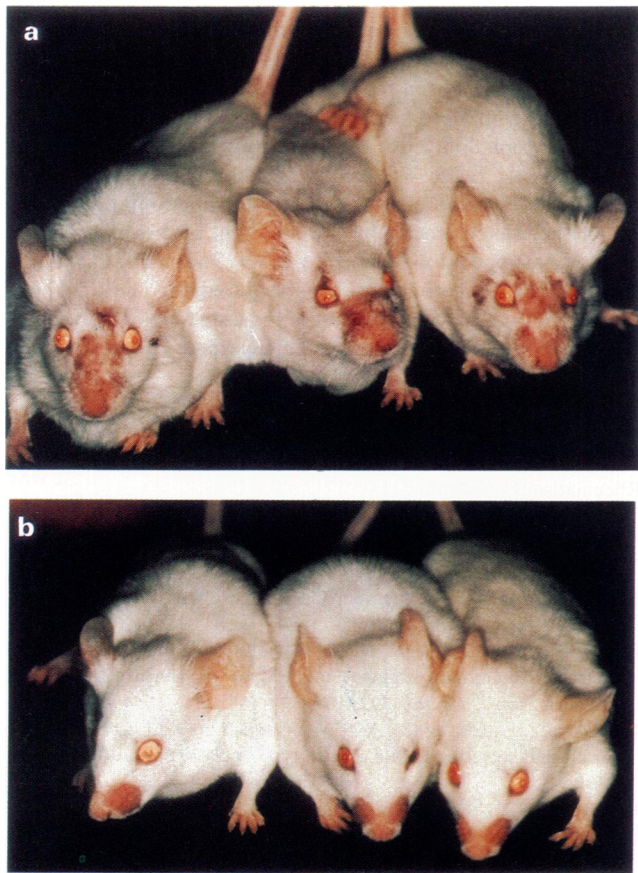
#### Effect of HBO on the spontaneous B cell activation in autoimmune NZB mice

In a previous report (Saito *et al.*, 1991) we showed that B cells from autoimmune NZB mice were spontaneously activated and differentiated into immunoglobulin-producing cells without any

**Table 1.** Effect of long term HBO exposure on MRL/lpr mice

	Four months survival (%)	Body weight (g)	Spleen cells ( $\times 10^6$ )	Lymph node (mg)	Proteinuria (score)	IgG-PFC (per $10^5$ spleen cells)	Anti-nuclear antibodies (OD units)
Control	70	42.9 $\pm$ 0.5	4.91 $\pm$ 0.31	580 $\pm$ 43	2.3 $\pm$ 0.4	65.0 $\pm$ 4.7	0.89 $\pm$ 0.08
HBO	100	39.9* $\pm$ 1.1	4.56 $\pm$ 0.28	310* $\pm$ 33	1.6 $\pm$ 0.3	46.2* $\pm$ 2.4	0.90 $\pm$ 0.10

Two-month-old female MRL/lpr mice were exposed to HBO daily for 2 months. The autoimmune clinical signs and laboratory data were evaluated at the age of 4 months. Survival rate is the percentage of the survived mice. Spleen cell number was counted by a Trypan blue dye exclusion test. Bilateral inguinal lymph nodes were measured and the average weight is shown. Urine protein indicates the semiquantitative score determined by urostics. IgG-PFC was detected by a reverse haemolytic plaque assay. Anti-nuclear antibodies in the sera were detected by ELISA. The results are expressed as the mean  $\pm$  s.e.m. of 10 mice in each group. \* Statistically different from the control group ( $P < 0.05$ ).



**Fig. 5.** Two-month-old female MRL/lpr mice were exposed to HBO daily for 2 months. The photograph shows (a) the representative three mice of control and (b) HBO-treated groups at the age of 4 months.

stimulation. We studied whether HBO could suppress this spontaneous immunoglobulin production of NZB mice or not. Four-month-old female mice were exposed to HBO at 252.5 kPa for 1 h once a day and immunoglobulin production of spleen cells was detected by a reverse haemolytic plaque assay. As shown in Fig. 4, spontaneous immunoglobulin production of B cells from NZB mice was decreased with increasing duration of exposure to HBO and significant reduction of immunoglobulin production was already observed on day 5 (five times exposure).

*Effect of long term HBO exposure on autoimmune MRL/lpr mice*

Finally, we studied the effect of long term exposure of HBO on the development of autoimmune diseases. Two-month-old female MRL/lpr mice were exposed to HBO once a day for 2 months and the clinical and laboratory signs evaluated at the age of 4 months. As shown in Table 1, the percentage survival at 4 months was significantly higher in HBO-treated mice than control mice. The body weight of HBO-treated mice was slightly but significantly reduced. Lymphadenopathy looks mild in the HBO group compared with the control group (Fig. 5). Moreover, it is notable that the number of spleen cells and the urine protein measured by urostics were low in level in the HBO group, although it is not statistically significant. Spontaneous immunoglobulin production by spleen cells of MRL/lpr mice is reduced by HBO treatment. However, anti-nuclear antibodies in the sera and the immune complex deposition in the kidney were not reduced in the HBO-treated mice during this experimental period. As shown in Fig. 5, marked facial erythema was observed in 4-month-old mice. However it can not be observed at all in the HBO-treated mice. These results suggest that the HBO treatment improves autoimmune symptoms.

**DISCUSSION**

HBO was initially applied for the patients with CO intoxication. Thereafter, the treatment was extended to patients with various kinds of condition caused by peripheral or generalized hypoxia represented by acute blood loss, skin ulcer, occlusion of central retinal artery and so on. The main mechanism of the improvement of such pathological conditions was considered to be marked elevation of tissue oxygen concentration. On the other hand, it has been found that HBO also changes some other physiological conditions such as immune responses. Some investigators reported that HBO showed some effects on immunological responses *in vitro* or *in vivo* (Murphy *et al.* 1975; Babior, 1978; Jacob *et al.* 1978; Warren *et al.* 1978a). In this paper we studied the effect of HBO on immune responses in detail.

HBO treatment of sheep-erythrocyte-immunized mice markedly suppressed anti-sheep erythrocyte antibody production. The suppression of anti-sheep erythrocyte antibody response in HBO-treated mice was not a kinetic difference, because the suppression of the antibody response was always observed on day 5, 7 and 10 after the immunization. The exposure of mice to HBO in an early stage and later stage after the sheep erythrocyte

immunization revealed a similar suppressive activity, suggesting that HBO does not disturb a specific phase of immune response such as the antigen recognition step by lymphocytes or the proliferation and differentiation step into antibody-producing cells, but affects any stage of the whole immune response. HBO seems to give a suppressive effect on both B cells and helper T cells, because either B cells or T cells from HBO-treated mice could not fully interact with T cells and B cells from normal mice.

As regards the mechanism of the suppressive effect of HBO, several possibilities can be considered. First, the direct oxygen cytotoxicity may cause the death of lymphocytes or induction of lymphocytes to cytostatic phase. Komori *et al.* (1984) reported that administration of peroxide of lipid including arachidonic acid induced the suppression of immunoglobulin production against both T cell dependent and independent antigens. Moreover, Horffeld (1981) observed that LPS-induced spleen cell proliferation was suppressed in 100% O<sub>2</sub>-exposed mice and the suppression was removed by the administration of scavenger agents such as vitamin E and superoxide dismutase. Thus, it is conceivable that HBO exposure induces oxygenation of lipid on the surface of cell membranes and the oxidative lipid produced acts on the lymphocytes as a cytotoxic agent. Such an assumption makes it easy to explain the general suppression of the whole process of primary PFC response against sheep erythrocytes and suppression of both T and B cells. But such cell injury seems to be relatively mild because the number of spleen cells was not markedly reduced in HBO-treated mice. Second, the oxidative stress may induce endogenous steroid hormone which acts as an immunosuppressive agent. In fact, adrenal hyperplasia and elevation of serum steroid was observed in HBO-treated rat (Bean & Smith, 1952). However, this seems insufficient to explain the immune suppression observed in this report because such elevation of steroid is only temporary and normalized in a few hours after HBO exposure (Houlihan, 1966). Thus, the mechanisms of immunosuppression by HBO require further experimentation.

Next, we tried to expose two kinds of autoimmune mice to HBO. NZB mice are one of several strains that develop a lupus syndrome comparable to that observed in humans. These mice are characterized by the presence of polyclonal B cell activation which results in the production of autoantibodies to DNA, erythrocytes and lymphocytes (Izui, McConahey & Dixon, 1978; Theofilopoulos *et al.*, 1982). MRL/lpr mice are also well known autoimmune mice. However, B cell hyperactivity in this strain is considered to be caused mainly by the overproduction of B cell differentiation factors by abnormal T cell subset (Steinberg *et al.* 1980; Prud'homme *et al.*, 1983a, b). We demonstrated that immunoglobulin production of both strains was suppressed by HBO. These results suggest that HBO affects both B and T cells, as we found in the experimental system using sheep erythrocyte-immunized C3H mice. However, we did not observe the reduction of anti-nuclear antibodies in the sera and the immune complex deposition in the kidney in the HBO-treated group during the time of the experiment. The discrepancy between the change of the cellular components and of the humoral components may be explained by the sensitivity difference of the detection method. Alternatively, it may be caused by the difference of the turnover rate between the cellular and humoral components. Since the turnover rate of the humoral components seems to be slower than that of the cellular

components, the decrease of immunoglobulin-producing cells may not directly reflect the change of the humoral components during the experimental period. We are now studying the change of humoral components in detail by using a large number of mice and by increasing the duration of the experiment. Of special interest is the fact that long term HBO can improve the survival of mice and autoimmune symptoms including facial erythema, proteinuria and lymphadenopathy. It has been observed that marked deposition of immune complex in the glomeruli of lupus mice (Lambert & Dixon, 1968) and tissue injury is caused by superoxide produced by macrophages and mesangium cells during the process of treating such a complex (Sedor, Carey & Emancipator, 1987; Warren *et al.*, 1987). HBO may induce the superoxide production by macrophages which results in tissue injury. However, the protective factors such as generalized suppression of immune responses and the production and continuous elevation of superoxide dismutase followed by the elevation of superoxide would overcome the disadvantageous factors induced by HBO. In addition, it has been reported that HBO suppressed exogenous antigen-induced autoimmune diseases such as experimental encephalomyelitis or adjuvant arthritis in the rat (Warren *et al.*, 1978b, 1979). However, this is the first report to show that HBO suppresses the progression of autoimmune signs which is restricted genetically. Although the precise mechanisms of immunosuppression induced by HBO remain unresolved and more detailed experimentation is required for clinical application, the evidence presented here suggests the possibility that HBO therapy is applicable for the treatment of autoimmune diseases.

#### ACKNOWLEDGMENT

This work was supported in part by a grant-in-aid from the Japan Rheumatism Foundation.

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