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OXYGEN IMMUNOSUPPRESSION: MODIFICATION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN RODENTS¹

JOEL WARREN, MIRIAM R. SACKSTEDER, AND CLAIRE ANN THUNING

From the Leo Goodwin Institute for Cancer Research, Nova University, 3301 College Avenue, Fort Lauderdale, Florida 33314

Using protocols that incorporated double blind examination of animals sensitized to CNS antigen, we confirmed and amplified earlier findings of the complete suppression of EAE in rodents by hyperbaric oxygen. The effects of O₂ were related to the gas pressure and duration of treatment. The development of paralytic disease was prevented for 34 days after sensitization (the longest interval studied). Compressed air or normobaric O₂ administered under similar conditions did not modify the course of illness. Within 7 to 10 days after the discontinuance of oxygen therapy the majority of treated guinea pigs developed typical signs of EAE with characteristic lesions in the CNS. The relapses occurred sooner in the Lewis rat. The development of the delayed hypersensitivity reaction to myelin basic protein and to tuberculin is also suppressed by O₂ therapy indicating that its effects upon autoimmune encephalomyelitis involves fundamental alterations of the cellular components of the immune response, some or all of which are reversible.

Investigations conducted over the past 3 years have demonstrated that prolonged exposure of rodents to hyperbaric oxygen results in the suppression of several cell-mediated immune phenomena including allergic encephalomyelitis (1), the tuberculin reaction, adjuvant disease (2), and allograft rejection (3). Here we amplify our preliminary observations on experimental allergic encephalomyelitis (EAE)² in the guinea pig and rat. This acute, cell-mediated autoimmune syndrome has been widely used as a research model for multiple sclerosis and its neurologic and vascular characteristics are well described (4). Because the clinical and histologic signs of EAE can be completely prevented when oxygen therapy is started after sensitization of lymphocytes has occurred, this phenomenon may prove useful as a probe for the study of delayed hypersensitivity

and as a therapeutic modality in cell-mediated autoimmune disease.

MATERIALS AND METHODS

Animals. Female, 450 to 500 g, outbred, Hartley strain guinea pigs (Buckberg Lab Animals, Inc. and Camm Research Institute Wayne, N. J.) were used. Lewis rats of both sex (Microbiological Associates, Bethesda, Md.) weighed 150 to 200 g at the time of sensitization. Animals were maintained on laboratory animal chow (Purina) and water. Paralyzed guinea pigs were hand fed as necessary.

Antigens. Frozen guinea pig spinal cord (Pel-Freez, Rogers, Ark.) was homogenized in physiologic saline, then emulsified with an equal volume of complete Freund adjuvant, CFA (Difco, Detroit, Mich.). Additional killed *Mycobacterium tuberculosis* was added so that the final preparation contained 25 mg wet wt. cord extract and 0.5 mg *M. tuberculosis* per dose of 0.1 ml. Fresh antigen preparations consistently produced 90% clinical disease and at least an 80% mortality in guinea pigs inoculated intradermally into the sole pad of one hind foot.

For the measurement of dermal sensitivity, myelin basic protein (MP) from guinea pig brain was diluted in physiologic saline solution and 0.1 ml was injected intradermally at concentrations of 5, 25, and 50 µg. Tuberculin, first strength, (PPD, Parke Davis, Detroit, Mich.) was used at a concentration of 5 µg/0.1 ml in saline solution. The indurated wheal on shaved dorsal skin was measured with a caliper 24 hr after injection. A response to BP with an average diameter of 0.5 cm or greater accompanied by erythema was considered as positive. The severity of the tuberculin reactions was compared on the basis of lesions of any size.

Scoring of clinical encephalomyelitis. A grading system was used wherein mild ataxia = 1, partial paralysis of one or both hind legs = 2, complete paralysis of one or more extremities and usually associated with incontinence and posterior soiling = 3. The mean daily indices were plotted over the duration of clinical disease. Animal cages were coded and the individual grading the disease was unaware of the treatment received by a specific group.

Preparation of material for histologic examination. Animals were killed with chloroform, and brain, portions of the encased lumbar and thoracic spinal cord, lung, and spleen were fixed in 10% formaldehyde. Sagittal sections of the thalamus, mid-brain, cerebellum, and pons and longitudinal sections of the spinal cord were stained with hematoxylin and eosin or in some cases with Luxol fast blue. In certain experiments, the inoculated foot was sectioned longitudinally and similarly stained. Perivascular, inflammatory lesions of the central nervous system were scored on the basis of their intensity and number.

Exposure to hyperbaric oxygen. (HBO). Animals, caged in

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² Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; CNS, central nervous system; HBO, hyperbaric oxygen; MP, purified basic myelin protein; PPD, purified protein derivative of tuberculin; DHR, delayed hypersensitivity response; RES, reticulo-endothelial system; MIF, migration inhibition factor; ATA, atmospheres, absolute.

TABLE I
Suppression of EAE in the guinea pig by HBO at 2 ATA

Oxygen Treatment			Median Onset of EAE		Clinical Signs ^a		CNS Lesions	
Start ^b	Stop	Total	Control	O ₂	Control	O ₂	Control	O ₂
		days						
+1	+10	10	10.5	17.5	12/12	0/11	ND	ND
+1	+14	14	10.2	27	8/8	0/10	ND	ND
+1	+17	17	10.5	29.3	73/85	0/77	35/35 ^c	1/38 ^c
+1	+34 ^d	17	9.0	48	7/7	0/9	ND	ND
+6	+14	8	9.6	12	7/8	0/9	5/5	1/4
+6	+23	17	12.5	27.5	20/30	0/25	ND	ND
+10	+14	5	11	11	17/18	14/16	9/9	7/7

^a Paralysis appearing during O₂ treatment.

^b O₂ started day 0.

^c Animals sacrificed on day 18 for histologic examination.

^d O₂ administered on alternate days.

groups of 6 to 10 were exposed to HBO in steel pressure chambers fitted with observation windows, loading ports, a floor plate, and a tray holding silica gel as the moisture absorbent.³

Oxygen tanks, set to deliver 99% O₂ at 100 psi, fed purge gas into the chambers at 5 psi. After 10 min of flushing, the exhaust valve was closed and oxygen flow was adjusted so that the rate of compression to the preset limit never exceeded 1 to 2 psi/min. The tanks were decompressed at a slightly slower (0.5 to 1.0 psi/min.) rate. Food and water were withheld from both control and oxygen-treated groups during periods of treatment.

Design of experiments. Rodents were sensitized to spinal cord tissue in CFA and one-half of each group was exposed to oxygen or compressed air at various pressures at different intervals before or after the inoculation of antigen. Treatment was continued for varying periods extending up to 5 weeks. As indices of suppression of EAE, we have used median time of onset, the extent and severity of clinical signs, histopathologic lesions in the central nervous system (CNS), mortality and survival time, and the response to the dermal injection of MP or PPD.

RESULTS

Suppression and reappearance of encephalomyelitis in the guinea pig. When guinea pigs were kept in oxygen daily at a pressure of 2 atmospheres absolute (2 ATA, 1520 mm Hg or 15 psi) for a period of 6 hr beginning 1 day after vaccination with cord-CFA emulsions and continuing for at least 10 days, the development of EAE was completely suppressed and the animals remained symptom-free for as long as oxygen was continued. In nine experiments involving 107 guinea pigs, none developed EAE during this period, whereas in the untreated controls, the incidence of encephalomyelitis was 100/112 (Table I).

When therapy was delayed for 6 days, HBO was still able to suppress completely EAE in each of 34 guinea pigs for as long as oxygen was continued. If HBO was not started until symptoms had begun to appear, day 10, it prolonged the median survival time over that of the controls but did not block the progression of disease.

Animals that received HBO and failed to develop encephalomyelitic signs had none of the pathologic CNS changes that characterize EAE. When 35 control rodents in three consecutive

³ Details of construction of the hyperbaric chambers will be provided upon request.

experiments were sacrificed 18 days after sensitization, all had typical, extensive perivascular and glial lesions in sections of the brain and spinal cord. In contrast, similar lesions were present in only one of 38 oxygen-treated guinea pigs that were symptom-free at this time.

Seven to 10 days after the discontinuance of hyperbaric therapy, typical signs of EAE appeared in the oxygen-treated guinea pigs. The lesions were identical in appearance to those that occurred earlier in the untreated controls and there was a similar degree of perivascular infiltration although the severity of illness was frequently decreased (see below).

Suppression and reappearance of encephalomyelitis in the Lewis rat. Oxygen immunosuppression of EAE was also demonstrable in the Lewis rat. However, the duration of suppression was shorter, being delayed in the rat for only 4 to 8 days, and eventually appeared in rodents undergoing daily oxygen treatment. Although a high incidence of clinical disease (37 of 40) developed in the controls, cases of fatal EAE were rare (4 of 40). Continued HBO invariably resulted in a significant amelioration of paralytic illness. A typical experiment in which the effects of hyperbaric and normobaric oxygen are compared is shown in Figure 1.

Toxic effects of prolonged HBO in rodents. Lewis and Fischer 344 rats and several strains of mice were exposed to O₂ at 2 ATA daily for 3 weeks or more without clinical evidence of toxicity. However, prolonged exposure of guinea pigs to HBO resulted in oxygen poisoning in a significant proportion (75 to 90%) of the animals. This rarely appeared before 10 days of treatment and was associated with rapid loss of weight and appetite and an increased respiration rate. When death occurred, it was often sudden and with signs of a respiratory block caused by extensive hemorrhagic atelectasis and a diffuse pneumonia as has been described by others (5).

Relation of the duration and initiation of oxygen exposure to suppression of EAE in the guinea pig. Two critical factors in oxygen immunosuppression were the duration of repeated exposures and the time at which oxygen treatment was initiated in relation to the inoculation of antigen. The reduction in paralytic disease was roughly proportional to the number of daily, 6-hr treatments and maximal when oxygen was begun 1 day after sensitization and continued for at least 10 days. Figure 2 illustrates the chronological relationship between initiation of treatment, onset and severity of EAE and occurrence of fatal oxygen toxicity in a large group of animals sensitized at the same time. The pattern of encephalomyelitis in five of six

untreated animals (line 1) is typical of a larger series of 184 untreated, sensitized guinea pigs. The course of disease was not significantly altered when oxygen was administered to healthy guinea pigs 7 days before and through 1 day before the injection of cord antigen (line 2). As the period of HBO treatment was extended to 2 weeks or longer (lines 3 to 7), and particularly if initiated 24 hr after sensitization, the incubation time to paralytic disease was lengthened. Even when therapy was begun as late as 1 day before the appearance of clinical signs (line 7), it modified the survival pattern.

Effects of interrupted HBO therapy on EAE. One measure proven effective in man as a means of reducing oxygen toxicity is the intermittent interruption of oxygen treatment by exposure to a normal inspired pO_2 . Suppression and significant amelioration of EAE, but without fatal oxygen poisoning, also could be achieved in the guinea pig by this procedure. The results of such an experiment are shown in Figure 3. All of the untreated animals had succumbed by the 12th day. When oxygen was administered daily for 17 days, 6 of 10 animals died of oxygen-induced pneumonia before day 25, three had EAE and one remained symptom-free for more than 100 days. In contrast, none of 10 guinea pigs given oxygen every other day developed pneumonia, three died of EAE (onsets on days 17, 48, and 48), four were paralyzed but survived, and three remain symptom-free for longer than 100 days. Similar results have been obtained in adjuvant disease of rats treated with O_2 on alternate days (2).

Relation of oxygen pressure to immunosuppression. A series of experiments has been performed in which guinea pigs or rats with EAE were exposed to pressures of 3 and 4 ATA (2280 mm Hg). At these higher pressures, the duration of daily treatment was necessarily limited to 1 hr. Table II summarizes results when HBO was started 1 or 4 days after sensitization. When treatment was continued for 18 or 24 days, there was a

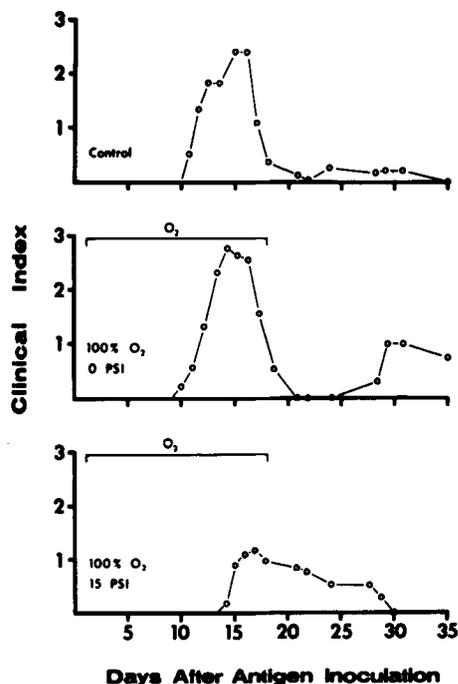


Figure 1. Effect of HBO and normobaric oxygen on EAE in Lewis rats. Groups of 10 sensitized animals were treated for 18 days with O_2 at pressures of 1 (normobaric) or 2 ATA. A similar number of rodents served as controls. The onset of paralysis was delayed by 4 days and the median clinical severity reduced by HBO. Oxygen (99%) at 1 ATA did not modify the course of disease.

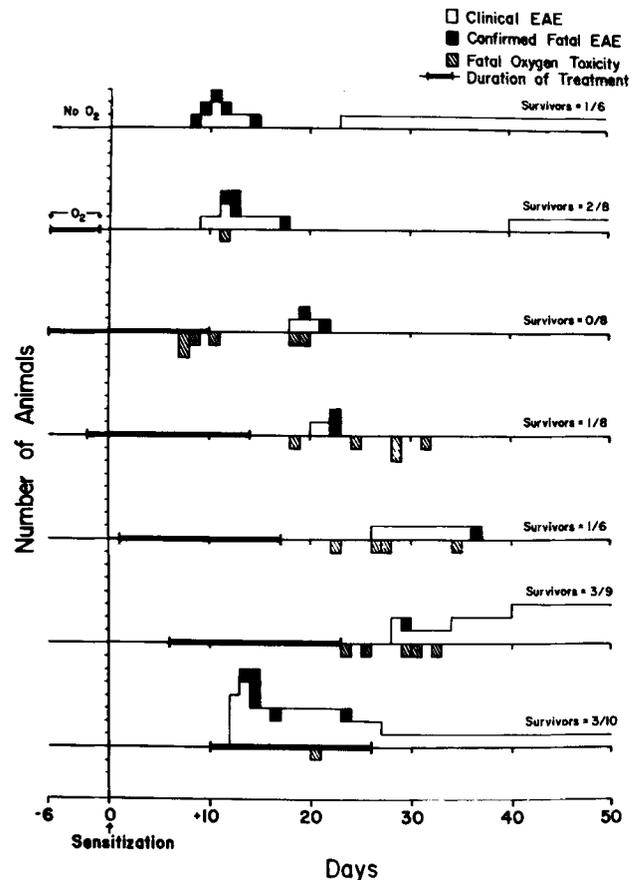


Figure 2. Relation of initiation and duration of oxygen treatment at 2 ATA to clinical EAE in the guinea pig. The onset and incidence of EAE and fatal oxygen poisoning is modified with the duration of therapy after, and not before, sensitization. When oxygen treatment was initiated as late as the 10th day, when symptoms first appeared, it still modified the pattern of illness as compared with that in untreated controls. Fatal O_2 poisoning developed in eight animals before the appearance of EAE in this study. None of these had histologic evidence of encephalopathy at that time.

consistent, small prolongation of the median survival time and a reduction in the severity of illness but no increase in the survival rate. As daily treatment progressed at these higher pressures all animals developed signs of pneumonia, and many succumbed with both EAE and oxygen-induced respiratory disease.

Normobaric pure oxygen or compressed air at 2 ATA will not suppress EAE. The effects of HBO could not be duplicated in sensitized rodents exposed to either normobaric air, compressed air at 2 ATA, or oxygen at 1.5 ATA in chambers under identical conditions (Table III). Although paralytic disease was less severe in the group given oxygen at 1.5 ATA, these findings suggest that a threshold for sustained elevation of the tissue pO_2 is required for complete inhibition of delayed hypersensitivity and that hyperbaric pressure *per se* is not a factor.

Suppression of the dermal response to antigens in the vaccine. The development of skin reactivity to MP and PPD in guinea pigs reaches a maximum at about 10 days and almost simultaneously with the onset of clinical encephalomyelitis (6). Oxygen-treated animals did not respond to the intradermal injection of 5 to 50 μg of MP when tested on the 7th day. Both of these immunologic events failed to appear by the 10th day in animals continuously treated from the 1st day after the injection of antigen-adjuvant emulsion, and tuberculin responsive-

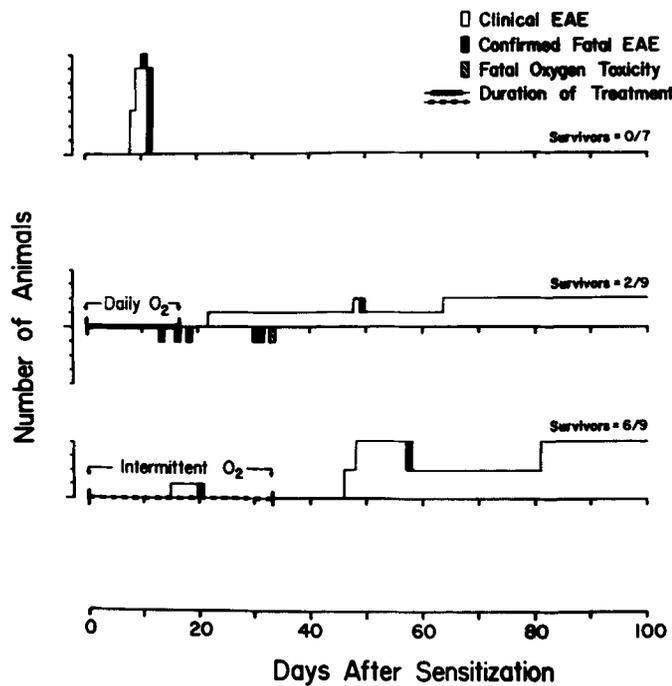


Figure 3. Effect of daily or intermittent oxygen treatment at 2 ATA on clinical EAE in the guinea pig. All untreated controls succumbed by the 11th day after sensitization. When O₂ was administered daily for 18 days beginning 1 day after sensitization, six of nine animals died of O₂ poisoning (no CNS signs) within 34 days; one died of EAE and two survived with posterior paralysis for 100 days. In contrast, when the same amount of O₂ was administered on alternate days, O₂ toxicity was avoided, three died of EAE, four developed moderate paralysis, and two remained symptom-free.

ness was suppressed after 17 days of therapy (Table IV). Delayed hypersensitivity to PPD reappeared 1 week after the return of each of four guinea pigs to air breathing.

Suppression of the granulomatous response to CFA antigen. The earliest clinical evidence of the suppressive effect of oxygen on EAE was marked reduction in the inflammatory response to the CNS antigen-adjuvant mixtures inoculated into the footpad. After 4 to 6 days of therapy, when there was extensive swelling and inflammation of the foot and ankle of the untreated animals, those receiving oxygen had only a slight, pale enlargement of the palmar surface. Histologic sections of the foot and ankle joint made after 18 days of oxygen treatment revealed a corresponding decrease in the inflammatory exudate and associated mononuclear cell infiltration present in the footpads of untreated guinea pigs (Fig. 4). Compressed air treatment had no effect on the lesion indicating that vasocompression was not responsible for the regression of the adjuvant-induced granuloma.

DISCUSSION

To recapitulate briefly these findings, oxygen immunosuppression of EAE is a reversible process requiring prolonged exposure to relatively low pressures of pure oxygen for 6 to 10 days. The reactivation of delayed hypersensitivity responses (DHR) upon discontinuance of oxygen requires a similar interval.

Optimum suppression of EAE was obtained when therapy was started within 24 hr after vaccination with neuroantigen and was continued for 17 days or longer. When shorter intervals were employed or when oxygen was given before sensitization, the time to relapse after the return to air breathing was

TABLE II
Effect of prolonged, daily HBO at 3 and 4 ATA on EAE in the guinea pig and rat

Rodent	Expt.	Oxygen		Clinical Signs	Median Onset	Survival ^a Time	Survivors
		Pressure	Treatment				
Guinea Pig	A	—	None	9/14	12	17.6	4/14
		3 ATA	1-18	6/14	12	19.3	4/14
	B	—	None	7/10	11	18.7	3/10
		3 ATA	1-18	5/10	14	20.5	3/10
C	—	None	9/10	11	16.5	2/10	
	4 ATA	4-24	7/8	11	17.0	0/8	
Rat	D	—	None	9/10	10	24.0	8/10
		3 ATA	1-18	7/8	11	—	8/8

^a Survival time refers to animals with clinical EAE, calculated from time of sensitization.

TABLE II.
Effect of compressed air or oxygen at pressures <2 ATA on EAE

Rodent	Treatment Days 1 through 17	Median Onset	Clinical Signs	CNS Lesions	Survivors at 30 Days
Guinea pig	None	10	27/30	24/24	1/10
	Compressed air at 2 ATA	10	27/30	21/21	0/10
Guinea pig	None	10	8/10	10/10	Sacrificed, day 18
	Normobaric air, 1 ATA, in chamber	10	8/9	9/9	Sacrificed, day 18
Guinea pig	None	10	7/7	10/10	Sacrificed, day 18
	Oxygen at 1.5 ATA	13	7/8	7/7	Sacrificed, day 18
Lewis rat	None	11	10/10	ND	9/10
	Normobaric oxygen (1 ATA)	15	8/10	ND	6/9

shortened. A state of oxygen-induced nonresponsiveness to CNS antigen could be maintained for a considerable time by the continuation of oxygen therapy.

It is noteworthy that HBO modified EAE when treatment was initiated as late as 6 days after injection of antigen. By this time, mobilization and sensitization of lymphocytes is well advanced, vascular permeability of the capillaries in the CNS is increased, (7) the ability to transfer passively EAE by sensitized lymphocytes and dermal sensitivity to myelin antigen are demonstrable shortly thereafter (6). Thus, within 4 days, (i.e., from days 6 to 10), oxygen therapy had arrested the cell-mediated events leading to tissue damage. The reduction in the granulomatous response at the site of antigen deposition is a significant element in the effects of HBO. Even though droplets of oil emulsion (CFA) persisted at the site of inoculation, the surrounding infiltration of mononuclear cells was minimal. Not only did oxygen therapy completely suppress central nervous symptoms if delayed until 6 days after sensitization, but 21 of

25 (84%) animals treated at this time were still alive at 30 days or 12 days after returning to air breathing.

The total absence of clinical signs of encephalomyelitis in optimal oxygen immunosuppression is due to a block in the antigen directed, mononuclear infiltration of the CNA; perivascular infiltration and demyelination, the hallmarks of autoimmune encephalomyelitis, do not occur. The absence of even minimal glial invasion together with the failure of treated guinea pigs to develop MP sensitivity attests to the completeness of the block. Although similar immunosuppression is demonstrable in the Lewis rat, it differs in its duration and extent from that in the guinea pig. This may be due to differences in species susceptibility to O₂ (8) and/or to differences in the pathogenesis of EAE in the rat and guinea pig. Each of the foregoing phenomena suggests that it involves interference with mobilization of the host inflammatory response but not T cell sensitization to myelin antigen. This hypothesis was strengthened by a recently completed preliminary passive transfer experiment with myelin sensitized rat lymphocytes. The development of histologic lesions of EAE was completely blocked in HBO-treated recipients and not in the untreated control rats. (These studies, performed in collaboration with Dr. P. Y. Paterson, will be described elsewhere.

In an earlier investigation by Prockop on the course of EAE in Hartley strain guinea pigs treated with oxygen at 3 ATA, daily 2-hr exposures on the 7th to 16th days post-sensitization prolonged survival by 1 to 2 days (personal communication). With the exception of this observation, oxygen immunosuppression *in vivo* does not appear to have been reported.

Two nonexclusive mechanisms whereby HBO could modify cell-mediated responses are under consideration: a direct effect of oxygen on one or more cell populations and/or an indirect result of endogenous steroids induced by the stress of hyperoxigenemia.

Several reports describe the detrimental effects of O₂ on phagocytic cells *in vitro* (9-11). We have speculated that this may be related to the intracellular reduction of oxygen to superoxide (O₂⁻) and H₂O₂, both of which have been suggested as key factors in bactericidal activity of phagocytes (12). The

TABLE IV

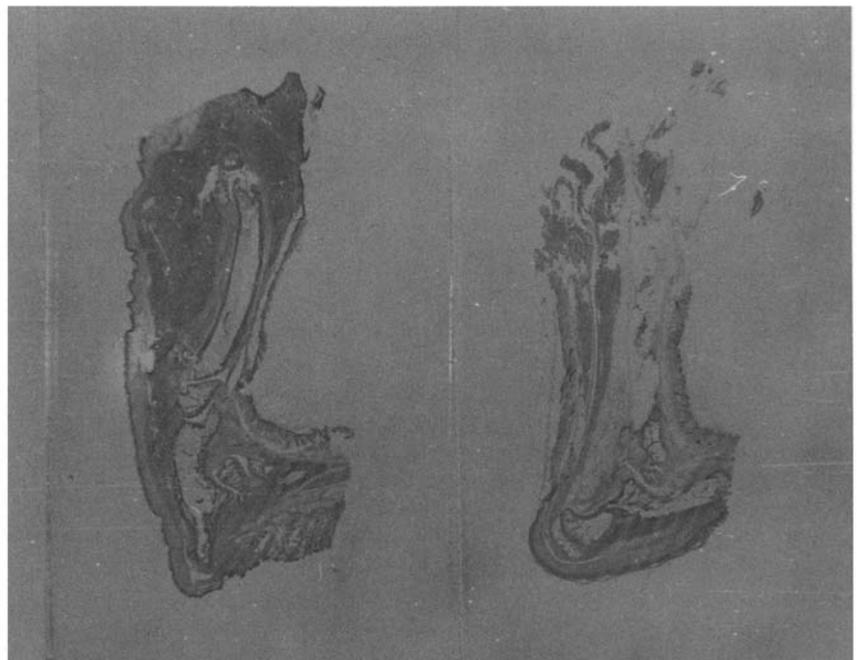
Suppression of delayed hypersensitivity response to M.P. and to tuberculin following prolonged exposure of guinea pigs to HBO

Treatment	Sensitizing Interval days	Reaction to			
		M.P. ^a , 50μg		PPD ^b , 5 μg	
		No.	Reaction	No.	Median diameter mm
None	7	3	3	N.D.	N.D.
O ₂ , days 1-7	7	3	0	N.D.	N.D.
None	10	5	3	4	1.48
O ₂ , days 1-10	10	5	0	2	0.4
None	17	N.D.	N.D.	2	1.46
O ₂ , days 1-17	17	N.D.	N.D.	2	0.5
None	28	N.D.	N.D.	5	2.14
O ₂ , days 14-28	28	N.D.	N.D.	5	1.66

^a For M.P., a wheal and erythema of 5 mm or greater was considered as a "positive reaction."

^b For PPD, the cross diameters of all reactions in the group were averaged.

Figure 4. Suppression of granuloma formation by HBO. Sections through the foot of guinea pigs sacrificed 18 days after the inoculation of guinea pig spinal cord emulsion in CFA into the pad. The section on the right was from an animal treated with HBO for 17 days beginning 1 day after sensitization. Although droplets of oil-antigen are still present in the subcutaneous tissues of the treated rodent, there is only a minimal granulomatous reaction as compared with the acute inflammation and monocytic infiltration in the control (× 2.5).



possibility that an increase in tissue pO_2 could result in superoxide levels in the activated macrophage that exceed the capacity of its protective oxidases is under study in our laboratory. Bean and Smith (13) observed that adrenocortical activity was stimulated by HBO, and that hypophysectomy diminished O_2 toxicity in rats. Adrenocortical hyperplasia and elevated serum levels of corticosterones occurred in rats exposed for 2 to 3 days at 1 atmosphere (14) and return to air breathing was followed by a drop in steroid levels within a few hours (15).

Exogenous corticosteroids are capable of suppressing essentially all DHR including EAE (16). A partial list of specific immunocomponents that are affected by steroids includes the uptake and digestion of particulates (17), the reduced ability of RES cells to destroy phagocytized bacteria (18), suppression of generation of sensitized cytotoxic lymphocytes (19), suppression of macrophage migration inhibitory factor (MIF) (20), and the loss of cortical thymocytes (21).

Certain characteristics of oxygen suppression of EAE resemble those of steroid immunosuppression, in particular the reduction of granulomata and the reactivation of inflammation after discontinuance of therapy. However, ascribing the effects of oxygen in blocking EAE to steroid activity requires resolution of certain inconsistencies. Pretreatment with HBO before sensitization (Fig. 2, Line 2), or exposure of guinea pigs to normobaric oxygen for 17 days after sensitization, procedures that one would expect to increase serum steroid levels, did not modify the course of EAE. Although the guinea pig is comparatively resistant to steroid effects, as measured by thymic depletion (22), O_2 completely suppresses EAE in this rodent. In contrast, encephalomyelitis in the rat, a corticosteroid sensitive species, was only delayed and "broke through" during HBO treatment. Clarification of the mechanism of oxygen immunosuppression and its possible relation to steroid physiology will require dissection of the effects of oxygen on various components of cellular and humoral immunity in parallel with measurement of its effects on adrenocortical activity.

It is appropriate to emphasize that our findings do not justify the immediate use of prolonged hyperbaric therapy in those human diseases presumed to have an autoimmune basis and multiple sclerosis, in particular. The lack of correlation between their clinical course and that of their experimental analogs in rodents, the hazards of prolonged, daily oxygen therapy, and, of greatest importance, our ignorance of the fundamental nature of the effects of oxygen on the immune response, all argue against any immediate application of these findings to prolonged, repeated HBO therapy of autoimmune disease in man.

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