

Effects of deep saturation diving on the lymphocyte subsets of healthy divers

N. SHINOMIYA, S. SUZUKI, A. HASHIMOTO, and H. OIWA

Department of Biology, National Defense Medical College, Tokorozawa 359, Undersea Medical Center, Yokosuka 239, and JMSDF Maritime Staff Office, Tokyo 107, Japan

Shinomiya N, Suzuki S, Hashimoto A, Oiwa H. Effects of deep saturation diving on the lymphocyte subsets of healthy divers. *Undersea Hyperbaric Med* 1994; 21(3):277-286.—We examined the effect of deep saturation diving on the host defense mechanisms of five healthy volunteers using fluorescein-dye-conjugated monoclonal antibodies. Six divers engaged in a 440-m saturation diving simulation with total hyperbaric exposure of 30 days; five served as subjects. Change in the expression of surface molecules on the lymphocytes was analyzed during that period. Blood samples were serially taken on Days 4, 6, 8, 15, 22, 29, and after surfacing. The total number of lymphocytes showed no remarkable change. However, the fraction of T (CD3⁺) cells decreased from $68.0 \pm 3.3\%$ to $55.8 \pm 5.8\%$ (Day 8), and B cells increased reciprocally. In these T cells, the CD4:CD8 ratio (normally >1.0) became less than 1.0 during compression and thereafter. In spite of the prophylactic use of anti-external otitis agents, one of the divers revealed a remarkable growth of *Pseudomonas* in the external auditory meatus, showing a high level of blood endotoxin (10.2 pg/ml). These results suggest that decrease in CD4⁺ fraction of T lymphocytes might explain in part the decreased resistance of divers to infective microorganisms in deep saturation diving.

saturation diving, lymphocyte subsets, CD4:CD8 ratio, host defense mechanism, immunosuppression

Hematologic changes related to various kinds of diving activities as well as hyperbaric oxygen (HBO) treatment have been reported by some investigators (1-6), and their results varied depending on the diving procedures. In a previous study, we reported that blastogenic response against phytohemagglutinin was remarkably suppressed after decompression of surface-based diving with various pressurizations (7). Several investigators attributed these functional and/or numerical changes of blood leukocytes to the immunosuppressive effects of exposure to HBO (3-6). These reports suggest that hematologic and/or immunologic changes in the divers are attributable to various environmental factors that are influenced by diving manner.

On the other hand, studies of the effects of saturation diving on the immune system are few (8), and this problem is still unsolved. In saturation diving, we often encounter infectious problems which are considered to be caused by increased susceptibility

and decreased resistance to microorganisms (9–11). For example, it is reported that a change in bacterial floras from *Staphylococcus* species to *Pseudomonas* species was observed as a result of the onset of otitis externa (12–14). These results may suggest that an immunosuppressed condition of the hosts is a serious problem in deep saturation diving. Nevertheless, host defense mechanisms during saturation diving are not well understood. Therefore, for safer diving operations it is necessary to clarify host defense mechanisms of saturation divers. This is one of the first studies of immunologic analysis in deep saturation diving from the viewpoint of changes in lymphocyte subsets that are closely related to the immunologic capacity of the hosts.

MATERIALS AND METHODS

Five healthy male divers ranging in age from 23 to 42 yr (mean \pm SD = 30.0 \pm 8.1 yr old) served as subjects.

A 440-m saturation diving simulation using a helium–oxygen gas mixture was carried out in a deep diving simulator at Undersea Medical Center (UMC), Yokosuka, Japan, in 1992. The diving profile and environmental conditions are shown in Fig. 1. To suppress the occurrence of severe high pressure nervous syndrome (HPNS), the

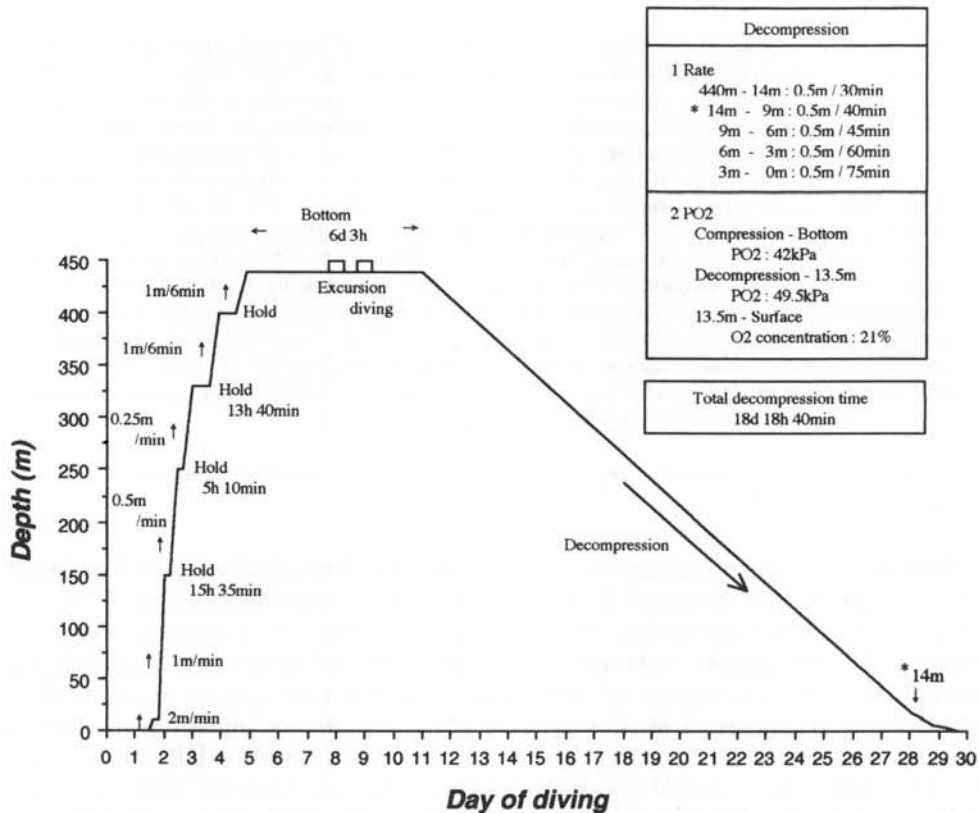


FIG. 1—Dive profile of 440-m saturation diving at UMC. Six men were exposed (five were served as volunteers) to compression to 440 m helium-oxygen. The rate of compression decreased with depths and pressure-hold stages were used to permit adaptation to HPNS. Decompression was performed according to the Duke-GKSS method kindly provided by Dr. R. Vann.

compression rate was gradually lowered as depth increased, and pressure was held at several stages. Decompression was performed according to the Duke-GKSS method (linear schedule; kindly provided by Dr. R.D. Vann, Duke University Medical Center, Durham, NC) with some modification. During the decompression phase, intravascular bubbles were not detected in any divers by an echocardiographic measurement.

Blood samples

Three milliliters of heparinized blood were collected from each diver between 0630 and 0700, before breakfast on the following days: once before the dive; on Days 4, 6, 8, 15, 22, and 29 of the hyperbaric exposure period; and after surfacing. To avoid possible membrane damage to cells in the blood samples, a decompression procedure to remove the samples from a hyperbaric chamber was managed carefully by decompressing a service lock at a rate of less than 10 meters of sea water (100 kPa) per minute. In addition, several 5-min-hold steps were made during the decompression procedure. Hematologic and biochemical changes were not affected by this sample recovery process. In this study, membrane integrity of the blood cells was well preserved, and we detected no cell damage using a flow cytometric analysis. Therefore, each lymphocyte subpopulation was considered to tolerate the decompression procedure equally well.

Monoclonal antibodies

Monoclonal antibodies (mAbs) that specifically bind to lymphocyte surface molecules (Table 1) and FACS lysing solution were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA).

Immunofluorescence staining and flowcytometric analysis

Whole peripheral blood was stained with fluorescein dye (FITC or PE)-conjugated mAbs at 4°C for 30 min. Then samples were treated with FACS lysing solution at room temperature for 10 min to lyse erythrocytes and to fix leukocytes. Subsequently, cells were washed twice and analyzed by a FACScan cytometer (Becton Dickinson

Table 1: Monoclonal Antibodies Used in This Study

Monoclonal Antibody		Lymphocyte Subset
FITC-conjugated	PE-conjugated	
1 Anti Leu-4 (clone SK7; CD3)	anti Leu-12 (clone 4G7; CD19)	pan T cells/B cells
2 Anti Leu-3a (clone SK3; CD4)	anti Leu-2a (clone SK1; CD8)	helper-inducer T cells/ suppressor-cytotoxic T cells
3 Anti Leu-7 (clone HNK-1)	anti Leu-2a (clone SK1; CD8)	natural killer cells/suppressor- cytotoxic T cells
4 Anti TCR- γ/δ -1 (clone 11F2)	anti Leu-4 (clone SK7; CD3)	γ/δ T cells/pan T cells

Immunocytometry Systems) using a 488 argon ion laser. Data were acquired using selective gating (forward light scatter and side scatter) and analyzed with Consort 30 software. Fraction of peripheral blood leukocytes was calculated by the scattergram that represents the cell volume and structure. By this method, peripheral blood leukocytes were clearly divided into three fractions: polymorphonuclear (PMN) leukocytes, lymphocytes, and monocytes.

Detection of bacteria

Bacterial floras in the external auditory meatus were examined by rapid bacterial identification systems. Briefly, ear samples wiped with a sterilized cotton swab were painted on ordinary agar plates. After an overnight incubation at 37°C, each colony was identified by Gram stain and selected by subsequent cultivation on Chapman 110 and NAC agar plates. The bacterial identification systems employed in this study were Staphygram and Nonfagram (Terumo Co. Ltd., Japan) for staphylococcal identification and non-fermentation bacteria such as *Pseudomonas* species, respectively.

Assay of endotoxin

For the measurement of serum endotoxin, an endotoxin-specific chromogenic test (Endospecky test; Seikagaku Kogyo) was used (15). Briefly, 100 μ l of sample serum was added to a 100- μ l portion of the test chemical dissolved in 0.2 M Tris-HCl buffer (pH 8.0), and the mixture was incubated at 37°C for 30 min. Absorbance was measured at 545 nm after diazotization. By this method, serum endotoxin levels of three divers were measured once before the dive; on Days 8, 15, and 22 of the hyperbaric exposure period; and 6 days after surfacing.

Statistical analysis

Results were expressed as the mean value \pm SD. Where appropriate, data were analyzed using repeated measures analysis of variance (ANOVA), followed by Dunnett's test for repeated comparisons. Values of *P* less than 0.05 were taken as significant.

RESULTS

From Days 2 to 6 (during the compression period), the number of peripheral blood leukocytes increased in three divers. This increase was transient, and leukocyte count returned to the normal range at Days 15–22. During decompression, the mean leukocyte number was kept almost at the same level as the value of prediving

(Fig. 2A). Although there were some differences in the pattern of leukocyte kinetics among individuals (Fig. 2B), such changes of leukocyte numbers were not statistically significant. From the viewpoint of leukocyte fraction, however, each fraction revealed characteristic changes (Fig. 3). The PMN population increased during the compression and bottom periods and gradually decreased through decompression. The lymphocyte population showed exactly the mirror image of the pattern of PMN. The monocyte fraction showed a decrease in the compression period. From the compression phase to bottom period, a slight increase in the total PMN was observed but was not significant. Moreover, the total number of lymphocytes showed no remarkable change; on the contrary, monocytes decreased in number as the depth increased.

Effects of saturation diving on the lymphocyte subsets were serially observed (Fig. 4). During the compression phase and bottom at 440 m, the fraction of T (Leu-4⁺; CD3⁺) lymphocytes decreased from 68.0% (before diving) to 55.8% (on Day 8), and that of B (Leu-12⁺) cells increased reciprocally (Fig. 4A). At this time, the percentage

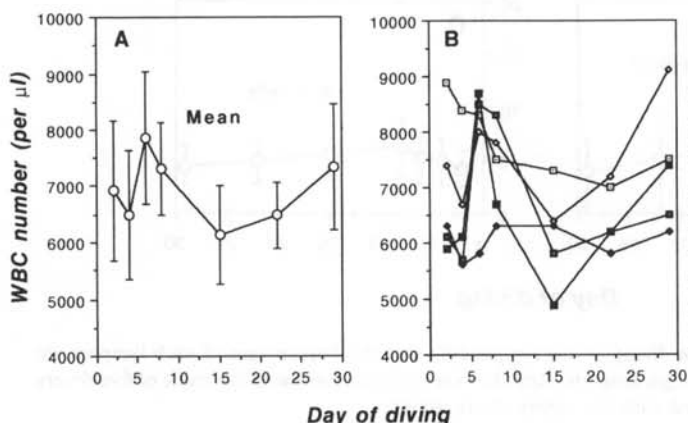


FIG. 2—Total number of peripheral blood leukocytes during 440-m saturation diving. Peripheral blood samples were treated with Zap-oglobin-II (hemolysing agent). Number of leukocytes were calculated with a particle counter. A, mean value \pm SD of five divers; B, individual results of five divers.

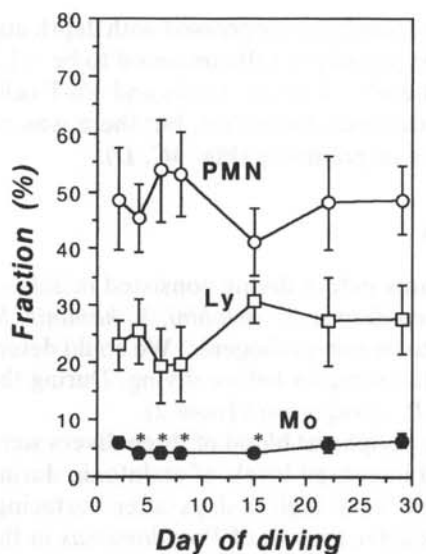


FIG. 3—Fraction of peripheral blood leukocytes during 440-m saturation diving. After treatment with FACS lysing solution, cells were analyzed with a cytometer. Each fraction of leukocytes was determined by light scatter analysis. Results were expressed as the mean value \pm SD of five divers. PMN: polymorphonuclear leukocytes, Ly: lymphocytes, Mo: monocytes. Asterisk $P < 0.05$, compared with the value of prediving.

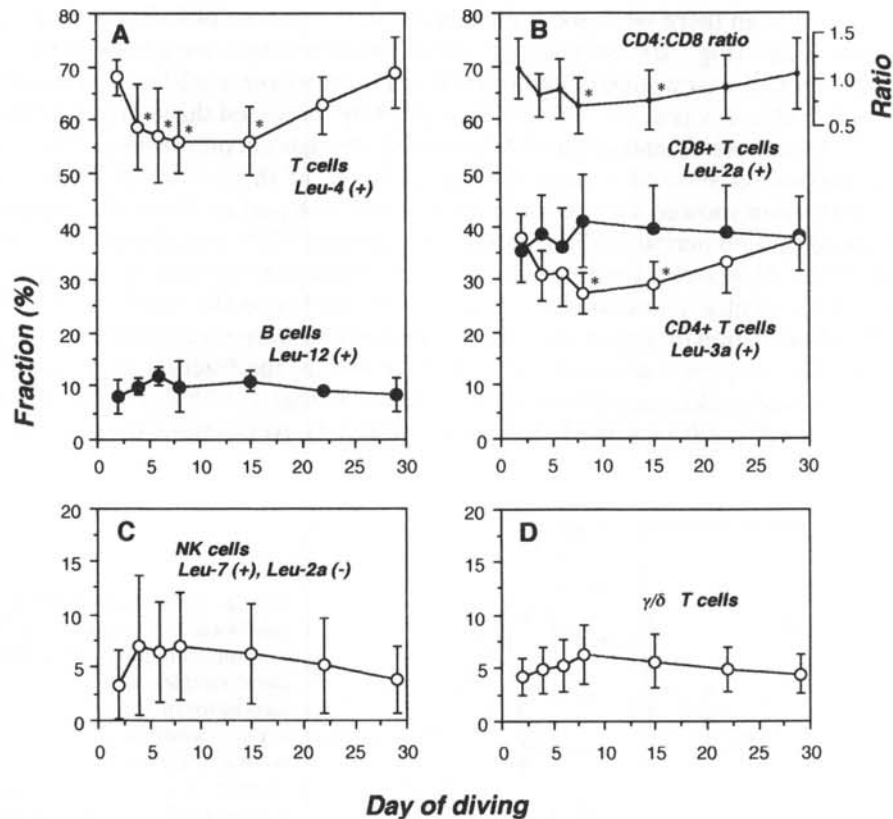


FIG. 4—Effects of 440-m saturation diving on the lymphocyte subsets. Percentages of each lymphocyte subset were calculated by contourgraph analysis. Results were expressed as the mean value of five divers \pm SD. Asterisk, $P < 0.05$, compared with the values of pre-diving.

of CD4⁺ (Leu-3a⁺; helper-inducer) cells was increasingly suppressed with depth and reached $27.3 \pm 3.8\%$ on Day 8. CD4:CD8 ratio (usually >1.0) continued to be <1.0 until surfacing (Fig. 4B). The fraction of NK (Leu-7⁺, Leu-2a⁻) cells and γ/δ T cells showed a tendency to rise until Day 8 and to decrease thereafter, but there was no significant difference compared with the values of prediving (Fig. 4C, D).

Bacterial floras in the external auditory meatus

Bacterial floras of the external auditory meatus before diving consisted mainly of *Staphylococcus* species such as *S. capitis*, *S. epidermis*, *S. delphini*, *S. hominis*, *S. caprae*. These bacteria are usually considered to be non-pathogenic. We could detect neither *Pseudomonas* species nor fungi from the samples before diving. During the dive, three of five divers showed positive for *P. aeruginosa* (Table 2).

Changes of the blood endotoxin levels in the peripheral blood of three divers were assayed (Table 2). One of three divers showed increased levels of endotoxin during the observation period and the value was still very high 6 days after surfacing, showing 10.2 pg/ml. This diver showed an extensive growth of *Pseudomonas* in the

Table 2: Bacterial Floras in the External Auditory Meatus and Endotoxin Concentration in the Peripheral Blood during Saturation Diving

Diver	Day of diving	Identified Bacteria, Colonies per Swab ^a		Endotoxin concentration, pg/ml
		Right ear	Left ear	
S. I.	Before	<i>S. caprae</i> (400) <i>S. aureus</i> (20)	<i>S. chromogenes</i> (100)	N.T. ^b
	Day 25	-	<i>P. aeruginosa</i> (>1,000)	N.T.
	Day 30	-	<i>P. aeruginosa</i> (4)	N.T.
S. M.	Before	N.D. ^c	N.D.	N.T.
T. K.	Before	<i>S. capitis</i> (150) <i>S. hominis</i> (100) <i>S. caprae</i> (10)	<i>S. capitis</i> (250) <i>S. delphini</i> (11)	< 5.0
	Day 8	-	-	< 5.0
	Day 15	-	-	< 5.0
	Day 22	-	-	< 5.0
	After 6 ^d	-	-	< 5.0
	M. T.	Before	<i>S. capitis</i> (150) <i>Staphylococcus spp.</i> ^e (300)	<i>S. delphini</i> (25)
Day 5		<i>P. aeruginosa</i> (120)	-	N.T.
Day 7		<i>P. aeruginosa</i> (>1,000)	-	N.T.
Day 8		-	-	< 5.0
Day 15		-	-	7.1
Day 22		-	-	5.0
After 6		-	-	10.2
N. I.		Before	<i>S. capitis</i> (150)	N.D.
	Day 8	-	-	< 5.0
	Day 15	<i>P. aeruginosa</i> (30)	-	5.0
	After 6	-	-	5.0

^aApproximate number of bacterial colonies observed per one swab was counted; ^bnot tested; not detectable; ^dsix days after surfacing; ^eprecise bacterial species could not be determined.

external auditory meatus. He complained of itching and pain, which are typical symptoms of external otitis.

DISCUSSION

Effects of various types of diving or hyperbaric exposure on the blood leukocytes, especially in the lymphoid system, have been reported by several investigators (1-5). However, the precise effect of the specified diving protocol on the immune system is still unclear and controversial. In this study, we investigated for the first time the effect of deep saturation diving on the immunologic capacities of healthy divers.

When divers were exposed to great depths, a considerable decrease in T cell subset, especially in CD4⁺ T cells, was observed (Fig. 4A, B) despite no significant

decrease in the total number of peripheral blood lymphocytes. The CD4:CD8 ratio rapidly decreased, reached less than 1.0 during the compression phase, and stayed at that low level until divers returned to the 1 atm environment (Fig. 4B). Change in the CD4:CD8 ratio and the depth seemed to correlate. Similar changes of lymphocyte subsets are sometimes observed in patients with an immunosuppressive state, such as acquired immunodeficiency syndrome (16). The CD4 antigens are expressed on the helper-inducer T lymphocytes; whereas CD8 antigens are expressed on the suppressor-cytotoxic T lymphocytes. CD4⁺ T cells play regulatory roles in the differentiation and activation of other lymphoid cells via cytokine production (17, 18) and induce effective immune reactions against infective microorganisms. CD4 molecules specifically bind to MHC class II antigens and activate various immunologic reactions through signal transduction pathways. Therefore, a rapid change in the CD4:CD8 ratio during deep saturation diving might affect the immunologic state of the divers.

During 440-m saturation diving, three of six divers tested positive for *P. aeruginosa* in the external auditory meatus. One of them complained of typical symptoms of external otitis, showing a high level of blood endotoxin (Table 2). It has been reported that the environment in the chamber, such as high temperature, high humidity, and limited ventilation, is the major cause of ear infection (19, 20). From our results, however, the existence of immunosuppressive mechanism in divers in the hyperbaric environment cannot be discounted. From the viewpoint of the difference between the infected and uninfected individuals, we found no significant correlation between the occurrence of otitis externa and lymphocyte subsets. Nevertheless, the CD4:CD8 ratio of peripheral blood lymphocytes during high pressure exposure revealed a low level in every diver. Change of lymphocyte subsets might explain the reason divers are highly susceptible to some types of microorganisms during saturation diving. As to the mechanism of decreased CD4:CD8 ratio, Bitterman et al. (6) reported that a significant increase was observed in the percentage and absolute number of CD8⁺ T cells, with a concomitant decrease in the CD4⁺ T cells immediately after HBO exposure.

Several studies have demonstrated that HBO has immunosuppressive effects on animals (3, 4) and these effects were attributable to the decrease in the number of lymphoid cells, including circulating leukocytes. However, the environmental conditions in saturation diving are quite different from those in HBO therapy. In this study, the maximal pressure was 4.5 MPa and the exposure time was 30 days, whereas in most HBO therapy, the total pressure does not exceed 280 kPa and the exposure time is no more than 2 h. Moreover, the oxygen partial pressure in this saturation diving was kept relatively lower (42–49.5 kPa) than that in HBO therapy. Therefore, high pressure rather than HBO is suspected as a main candidate inducing immunosuppression. B cells, NK cells, and γ/δ T cells showed a tendency to increase during the 440-m saturation diving (Fig. 4A, C, D) in contrast to a decrease in CD4⁺ T cells, whereas no changes in the total count and percentage of T cells, B cells, and NK cells were recognized in the HBO study (6). Similar changes in lymphocyte subsets were also observed in 100- and 330-m saturation dives conducted at UMC in 1993 (data not shown).

These results suggest that the mechanism of immunosuppression during saturation diving might be explained by rapid compression or high hydrostatic pressure. In addition, saturation divers are exposed to various kinds of neurologic and psychologic stresses such as HPNS, dense gas breathing, anxiety confinement in a sealed environ-

ment. During the compression phase, environmental factors change so rapidly that divers cannot adapt to a new environment, which may lead to unbalanced hormone/cytokine production. Some reports indicate that stress-induced corticosteroids down-regulate the expression of various cytokines (21–24). These results may cause selective depletion of the CD4⁺ T cells' lymphocyte subpopulation and subsequent alteration of the CD4:CD8 ratio and other lymphocyte subsets. As we are now measuring some cytokine levels during deep saturation dives (IL-2, IFN- γ , and others), we hope that the relationship between cytokine levels and lymphocyte subsets will be clarified in the near future. At present, however, this hypothesis is based on the limited data from one 440-m saturation dive.

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