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Effects of 30-m nitrox saturation dive on the immune system in man.

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Shimamiya T, Terada N, Wakabayashi S, Mohri M. Effects of 30-m nitrox saturation dive on the immune system in man. Undersea Hyperb Med 2006; 33(1):63-68. Hyperbaria reportedly affects the immune system, but the role of psychological factors arising from confinement has not been taken into consideration. We investigated the immune changes in 4 subjects exposed to a 9-day simulated 30-m (400-kPa) nitrogen-oxygen (nitrox) saturation dive, and compared the results with those of our previous study that showed immune and mood changes in normobaric confinement. Blood samples were taken before, during, and after the dive or confinement, and activated with an anti-CD2 agonistic antibody. The percentages of granulocytes, natural killer (NK) cells, and cells positive for CD69, an early activation marker, were analyzed by flow cytometry. Reduction of CD69 expression percentage was observed under both hyperbaric and normobaric conditions. Percentages of innate immune cells, such as granulocytes and NK cells decreased or remained mostly unchanged, contrasting with our previous study, which demonstrated increases in both percentages coordinate with mood improvement. We conclude that these changes may have been triggered by suppression of sympathetic nerve activity that occurs in 30-m nitrox saturation hyperbaria.

INTRODUCTION

Although different diving profiles have been used, the immune system has been reported to undergo changes in saturation diving. The suppression of proliferative responses of lymphocytes to phytohemagglutinin (PHA) has been observed in surface-based air diving and in animal experiments (1,2). Changes in the distributions of leukocyte subpopulations, such as neutrophils, monocytes, lymphocytes, and lymphocyte subpopulations, were also reported in several studies examining effects of hyperbaria on human physiology (1,3,4).

Because saturation diving entails confinement, some reports have suggested that these immune changes are partly due to the psychological stress of confinement (1,3,4). Psychological stress affects the human immune system, for example, in acute experiments that entailed a heart rate elevation, an increase was detected in NK cell number (5). Various reports have demonstrated a relationship between the immune system and stress of various types, not just psychological, but physical or environmental factors such as exercise, injury, temperature, gravity, and changes in gas pressures including hypo- and hyperbaria, oxygen (6,7). We previously conducted a 10-day confinement study in a non-pressurized diving simulator with 10 male volunteers and reported an increase in NK cell and granulocyte ratios and reduction in lymphocytes positive for CD69, an early activation marker of immune viability (8,9). Thus, immunological responses reported in hyperbaria may simply have been caused by the psychological stress of confinement.

The present study was performed to determine the effects of hyperbaria on the immune system by comparison with the data of our previous study (8,9). We analyzed the percentages of granulocytes, NK cells, and lymphocytes positive for CD69 in subjects participating in a 30-m (400-kPa) nitrox simulation dive.

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METHODS

The Japan Marine Science and Technology Center (JAMSTEC, Yokosuka, Japan) dive simulation chamber was used for the nitrox saturation dive in the present study. The simulator, measuring 34.1 m^2, was equipped with bunk beds and hygiene facilities. Four Japanese male divers (age, 23-37 yr; mean, 26.8 yr) participated in the study. The subjects underwent medical check-ups and psychological tests and were considered healthy. The same medical and mental check-up procedures were conducted in the normobaric confinement study we reported previously (8,9). Two of the subjects had saturation diving experience in the previous five years and two did not, and none of the four had participated in the normobaric confinement study. The experiment was conducted in November 1999 and lasted 18 days. The study protocol was approved in advance by the JAMSTEC human studies committee. The purpose and possible risks associated with the experiment were explained to each subject, all of whom provided written informed consent before participation. The subjects spent time in the chamber measuring physiological data and performed moderate ergometer exercise for 20 minutes per day. The schedule consisted of a 4-day pre-dive period, 10-day dive period, and 4-day post-dive period. Compression started at 10:00 on Day 5. The chamber was pressurized over 78 minutes to 4 atmospheres absolute (ATA), or 400 kPa (PO_2 = 40 kPa, PN_2 = 360 kPa and PCO_2 < 0.5 kPa), where relevant to below 30 m of seawater (msw). Chamber temperature was kept at 26°C during the dive period and at 24°C in the pre- and post-dive periods. Relative humidity was maintained at 60% throughout the experiment. Decompression began at 15:50 on Day 12, and the chamber was depressurized over a period of 52 h 10 min. Blood samples were taken into heparinized test tubes (Vacutainer, Becton Dickinson, Mountain View, CA) at 7:00 AM twice each in the pre-, inter-, and post-dive periods.

The blood samples were transported from Yokosuka to Yamanashi at 4°C. Six hours after the blood samples were drawn, 20 µg/ml CD2 agonistic antibody solution, CD2/CD2R (CD2: L303.1, CD2R: L304.1, Becton Dickinson), was added to the whole blood and the samples incubated for 4 h at 37°C. Samples without CD2/CDR were prepared as controls. CD2/CD2R contains two kinds of anti-CD2 antibody that recognize different epitopes in the CD2 molecule and specifically activate NK and T cells (10). Total leukocyte number was counted with a Sysmex microcell counter F-300 (Sysmex, Kobe, Japan).

Aliquots of 50 µl of activated blood were incubated with 20 µl of fluorochrome-labeled antibody cocktail solution (FASTImmune, Becton Dickinson) for 20 minutes. The solution included FITC-labeled anti-CD56 antibody (NCAM16.2), PE-labeled anti-CD69 antibody (L78), and PerCP-labeled anti-CD45 antibody (2D1). CD56, CD69, and CD45 antigens are specific surface markers expressed on NK cells, activated lymphocytes, and leukocytes, respectively. After hemolysis and fixation with 450 µl of FACS lysis solution (Becton Dickinson), samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Flow cytometry settings for the measurements were in accordance with the manufacturer's instructions. Cell Quest software (Becton Dickinson) was used for data acquisition and analysis. A total of 10,000 events, which passed a gate set on a lymphocyte subpopulation in a side scattering (SSC) *vs*. CD45 dot plot, were analyzed on a CD69 *vs*. CD56 dot plot. By referring to a non-activated sample, a quadrant marker separating the CD69- or CD56-positive subpopulation was set on the plot. NK cells (CD56+) are shown on the right, while non-NK cells, consisting of T and B cells, are shown on the left. In activated samples, CD69-positive cells were seen in the upper area. The four areas on the plot sum to

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100%. The percentage in each area was taken relative to the whole 10,000 events of CD45-positive lymphocytes gated at the SSC *vs*. CD45 plot. The population that emerged in the upper area of the FSC *vs*. SSC dot plot was gated and analyzed as granulocyte percentage.

Descriptive statistical analyses were performed for each time point. After multivariate analysis of variance (MANOVA) concerning each variable, Dunnett's multiple comparisons between the first time point (Day 2) and other time points were performed. Data are expressed as mean ± standard error (SE). JMP software (SAS Institute Inc., Cary, NC) was used for statistical analysis.

RESULTS

A reduction of CD69 expression percentage in non-NK cells was observed in the saturation dive period (A). This reduction lasted until after decompression. The granulocyte percentage relative to the whole leukocyte population decreased in the saturation dive period (B). No significant changes were observed in the percentage of NK cells relative to the whole lymphocyte population throughout the experiment (C). Although not statistically significant (P= 0.05) an increasing trend was observed at Day 7 with the day hatch closed and chamber compressed. The mean WBC counts per µl were 6,425±629, 6,325±739, 6,475±448, 6,075±548, 6,375±407, and 6,150±517 from the pre- to the post-dive period. WBC count showed no marked changes throughout the period of the experiment.

DISCUSSION

A potential limitation of the present data should be noted before the discussion. In this study, another group conducted adrenoreceptor sensitivity tests (11), which required intravenous administration of adrenergic agonists. These agents were administered just after our blood samples were taken. The subjects' heart rates were reported to change due to the administration, but returned to the basal levels shortly after each test. As our next samples were taken at least 24 h after administration of adrenergic agonists that are metabolized within minutes, we regarded their effects on our measurements as negligible.

CD69 is an early activation marker of lymphocytes expressed within 2-4 hours on the pathway to proliferation, and in a widely used proliferation assay, the expression of CD69 is correlated with ^{3}H-thymidine incorporation (12). Analysis of CD69 as a marker of proliferative responses in blood lymphocytes may provide results that reflect the effects of various stresses, as the data can be obtained while blood samples are still fresh and retain their *in vivo* status. Moreover, as flow cytometry is available to measure CD69 antigen expression, information can be obtained on a subpopulation basis. This enabled us to observe the relationships between the suppression of proliferation and distribution changes in lymphocytes.

Hyperbaria is known to affect immune parameters. Matsuo *et al*. reported changes in leukocyte and lymphocyte distributions, such as a decrease in CD4:CD8 ratio, as seen in patients positive for human immunodeficiency virus or mental stress, and a rise in NK cell ratio of about 10% while compressing to 400-m (4.1-MPa) with helium and oxygen (4,13). Philp *et al*. also reported 10-30% increases in leukocyte count from baseline during and after saturation dives to various depths from 60 fsw (0.28 MPa) to 300 fsw (1 MPa) (3). These changes were reported to be due to infections caused by the humid environment and inflammation caused by air bubbles generated during decompression (1,3,13). On the other hand, endocrine changes caused by psychological stress are known to trigger changes in leukocyte distribution. Experimental stress, such as arithmetic, public speaking, etc. that necessitates an increase in heart rate causes an increase in NK cell number of 1.5 fold relative to baseline (5). Leukocytes have receptors for stress hormones, such as catecholamines and corticoids, and

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their distribution and viability are controlled through those receptors (5,14,15). Stimulation of sympathetic nerves induced by stress_is thought to control rapid mobilization of innate immune cells, such as NK and granulocytes from the spleen and marginal pools, and to elevate the number of those innate immune cells, in peripheral blood vessels (15). Innate immune cells are also reported to have large numbers of catecholamine receptors (5,14,15).

In a previous 10-day confinement study of 10 healthy male subjects under normobaric conditions, we observed increases in percentages of NK cells and granulocytes toward the end of confinement (8). Interestingly, mood changes in subjects, assessed with the face scale test (16) were also elevated toward the end of the confinement study. Higher point scores in the face scale test indicate a better mood in the subjects. Thus, the subjects' mood became more delighted as the end of the experiment approached. Moreover, the subjects who showed significant increase in the face scale also showed significant rise in the percentages of granulocyte and NK cells (9). These data showed mood changes could relate to changes seen in innate immune cells. Elevation of sympathetic nervous activity in subjects with excited mood affected their endocrine status that may have resulted in increases in NK cell and granulocyte percentages.

In contrast to these previous data, the percentages of NK cells and granulocytes in the divers in the present study remained mostly unchanged or decreased (B, C in Fig 1). As little change was observed in WBC number throughout this experiment, the percentages should reflect the actual number of each subset. Bradycardia in divers suppresses sympathetic nervous activity entailing a reduction in basal heart rate (17). Thus, down-regulated sympathetic activity in the divers may contribute to the observed near lack of change or decrease in NK or granulocyte percentages. It is also possible that granulocyte distribution is more sensitive to hyperbaria than that of NK cells.

Seno *et al*. reported suppression of phytohemagglutinin (PHA)-induced lymphocyte proliferation in surface-based short-time air dives to 39.6 msw (500 kPa). However, it remains to be clarified which factors are related to the suppressive reaction in saturation diving (2). As saturation diving entails confinement and adverse conditions, many reports discuss the effects of psychological factors (1,3,4) but few normobaric studies have tried to exclude the possibility that observed effects are due to confinement alone. Nagashima *et al*. reported a decrease in total sleep time not only in hyperbaric, but also in normobaric experiments, and they suggested the involvement of stress factors due to confinement (18). As shown in this study, CD69 was suppressed in the saturation dive period (A in Fig. 1); however, a similar decrease in CD69 percentage was observed in a previous normobaric study (8). The reduction in CD69 percentage was significant in the subjects who showed significant mood changes (9). Thus, the observed reduction in CD69 percentage under hyperbaric conditions could have been induced by changes in mood. These results indicate the need for careful interpretation of outcomes obtained in saturation dive studies as the same phenomena may occur in simple confinement.

As saturation dives vary widely in terms of compression procedure, duration, and breathing gases, it is incorrect to expect the same immune response from dissimilar saturation conditions. Experience in saturation diving or confinement and personality traits should also be taken into account, as physical and psychological adaptation occurs in experienced individuals. An appropriate subject number and controls, e.g. a non-confinement time-matched group, should be utilized to fully determine the immune changes in saturation conditions. Although this study had some of these limitations in design, our observations do suggest that factors arising from confinement alone are significant in saturation diving.

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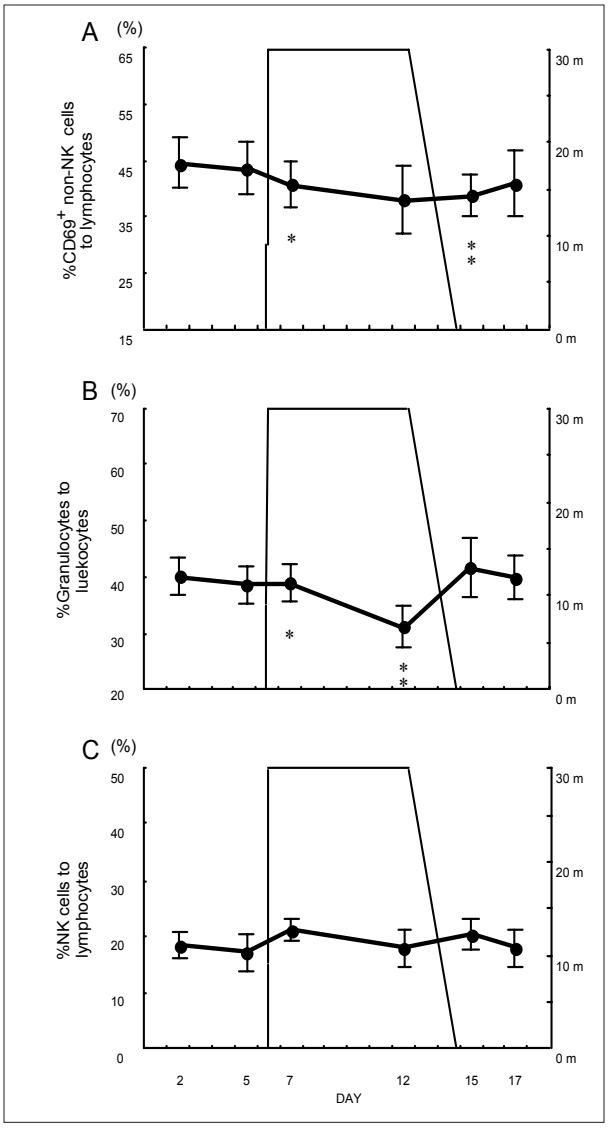

Fig. 1. Changes in immune parameters under hyperbaric pressure. From the top, percentages in lymphocytes positive for CD69 (A), granulocytes (B), and NK cells (C) are shown. he fine solid bar in each plot indicates depth. Values are means±SE. Significant differences between pre-dive and other time points: *P < 0.05; **P < 0.01 (n = 4).

In conclusion, expression of the early proliferation marker, CD69, was decreased in a human nitrox saturation dive to a depth of 30 m. As the same decrease had been observed in the normobaric confinement group in our previous study, factors such as the stress of confinement may have been involved in this reduction. The differences in reaction compared to the normobaric confinement study, such as a near absence of change or decrease in percentage of innate immune cells (*e.g.*, granulocytes and NK cells) during the dive period, may have been induced by the suppression of sympathetic activity in the saturation dive. These observations suggest that saturation divers might be susceptible to infections related to a shortage of granulocytes, and that countermeasures should perhaps be considered.

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