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Venous gas emboli and complement activation after deep repetitive air diving

J. ZHANG, C. E. FIFE, M. S. CURRIE, R. E. MOON, C. A. PIANTADOSI, and R. D. VANN

Departments of Medicine and Anesthesiology and F. G. Hall Hypo-Hyperbaric Center, Duke University Medical Center, Durham, North Carolina 27710

Zhang J, Fife CE, Currie MS, Moon RE, Piantadosi CA, Vann RD. Venous gas emboli and complement activation after deep repetitive air diving. Undersea Biomed Res 1991; 18(4):293–302.—Complement activity has been linked to decompression sickness (DCS), but the effects of intravascular bubbles on complement activation are poorly understood. We have investigated intravascular complement activation by measuring red blood cell (RBC)-bound C3d after repetitive air diving in man. Subjects were exposed to a single, 20 min, 170 fsw (feet of sea water) dive, or to 2 such dives with a 6-h surface interval. Doppler monitoring for venous gas emboli was performed postdive. Predive blood samples were studied to determine sensitivity of complement to activation by air bubbles. Other predive and postdive venous samples were evaluated for intravascular complement activation. No cases of DCS occurred in 39 dives. Baseline complement sensitivity appeared normally distributed, thus "sensitive" and "insensitive" subjects were not clearly distinguishable. RBC-bound C3d did not increase after 1 dive but did increase after the repetitive dive ($P < 0.05$). Furthermore, maximum bubble grade was independent of complement activation.

decompression sickness
Doppler monitoring
C3d assay

The development of low-risk decompression procedures would be enhanced by a more physiologically sensitive measure of decompression stress than the presence or absence of the symptoms of decompression sickness (DCS). Doppler ultrasonic bubble detection has been used in this capacity to monitor the incidence of circulating venous gas emboli (VGE), but the correlation between DCS symptoms and VGE is not entirely satisfactory (1–5). VGE frequently occur without DCS whereas DCS occasionally occurs with no detectable VGE (1, 6, 7).

It has been postulated that DCS results from hematologic interactions at blood-bubble interfaces, but the correlation between DCS symptoms and most of the hematologic parameters that have been studied is poor (8, 9). Recently, correlation between DCS and the effects of gas bubbles on blood complement proteins (9–14)

has suggested that DCS may be mediated by activated complement, subsequent inflammatory responses, and microvascular injury. Evidence supporting this hypothesis led Ward et al. to propose that individual variation in DCS susceptibility is a consequence of natural changes in complement sensitivity (13, 14). Ward and colleagues defined sensitive subjects as having a significantly greater C5a increase than insensitive subjects when their plasma was incubated in vitro with bubbles (13, 14). Sensitive divers and rabbits were observed to have a higher incidence of DCS. In contrast, rabbits that had been decomplemented shared both reduced complement sensitivity and lower DCS incidence.

These results are encouraging, but complement activation is a complex process (Fig. 1). Many factors can influence the formation and degradation of complement components, and it is not clear which of these proteins might be closely associated with DCS. The mechanisms of this association, moreover, are uncertain. In hopes of clarifying the relationship between complement activation and decompression stress, we have investigated the time course of intravascular complement activation before and after repetitive air diving.

Prerequisite to the study of intravascular complement activation was the identification of a relatively stable complement protein and an appropriate assay. As indicated in Fig. 1, complement C3 arises from both the alternate and classical pathways. C3 splits into C3a and C3b, and C3a and C5a have been proposed to contribute to DCS (13, 14). These components, however, are difficult to assay in vivo because they bind rapidly to tissue. Covalent attachment of the C3b fragment to target acceptors, such as red blood cells (RBCs), is required for complement activation to proceed because C3b provides the binding site for C5. Bound C3b can either initiate membrane attack or be degraded to fragments (C3c, C3d, etc., Fig. 1). Most C3d remains covalently bound to the cell surface, hence an increase in the RBC-bound C3d level would reflect the activation of C3. Assay of C3c has been used previously to investigate complement activation during several consecutive weeks of diving (15), but C3c is eliminated from plasma very rapidly and thus may not be a sensitive indicator of intravascular activation (16). The C3d fragment, the final cleavage product of C3, is relatively stable in vivo either on a target cell or in the fluid phase. Therefore, it is a good candidate for a marker of early activation of the intravascular complement cascade (16, 17).

We measured complement activation by assay of RBC-bound C3d and monitored circulating VGE in subjects exposed to single and repetitive deep air dives in a dry chamber. DCS was not expected and did not occur. Our objectives were to study the

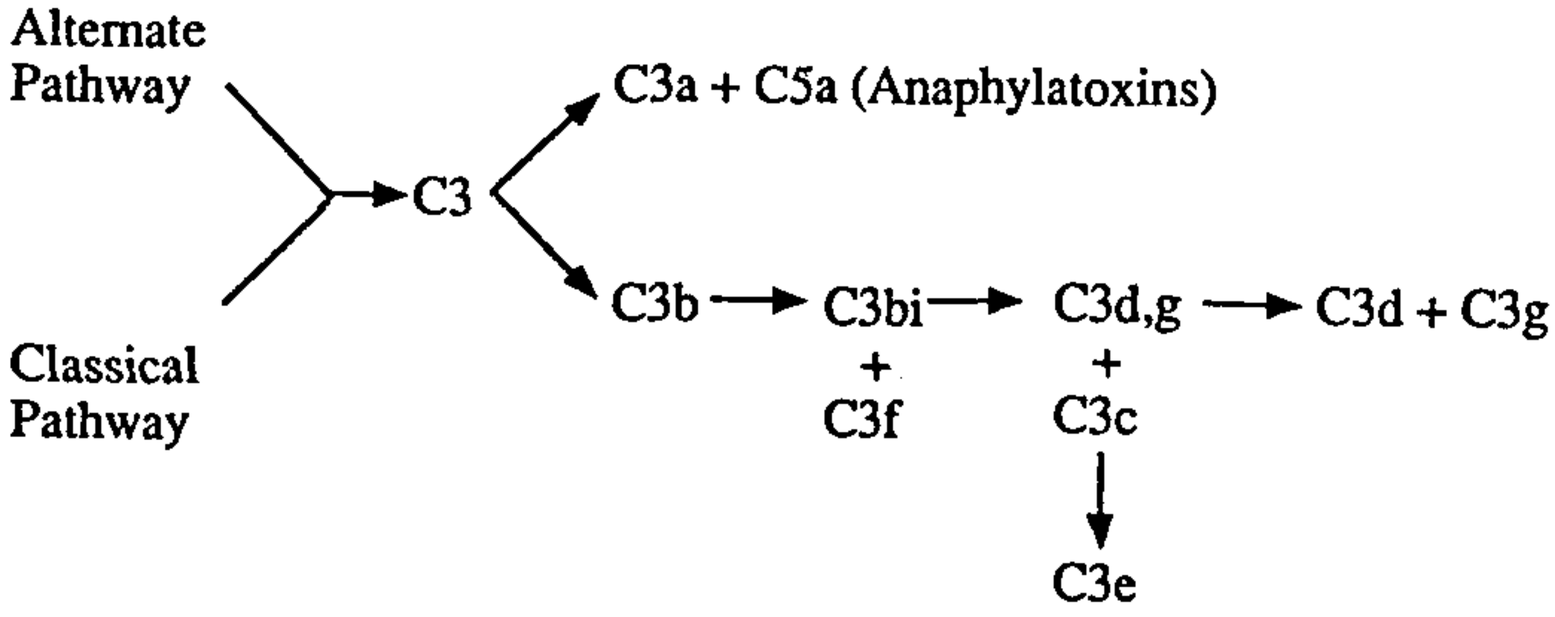

Fig. 1. Complement cascade. Complement C3 is a key protein which arises from both the alternate and classical pathways. C3d fragments is one of the final cleavage product of C3.

relationship between VGE and intravascular complement activation during repetitive diving and to determine if the RBC-bound C3d assay could detect differences in individual sensitivity to the effects of gas bubbles in a manner similar to that reported for assay of C5a (13, 14).

METHODS

The pressure chamber exposures and Doppler bubble monitoring took place at the F. G. Hall Hypo-Hyperbaric Center of Duke University Medical Center. Complement assays were performed at the Laboratory for the Study of Immuno-senescence of the Durham Veteran's Administration Hospital. The experimental procedures were approved by the Institutional Review Board of Duke Medical Center, and informed consent was obtained from all subjects.

Dive profile

The dive profile shown in Fig. 2 had been developed for a project by the Institute of Nautical Archeology (INA) (18). The repetitive air tables used were for depths of 150–180 fsw (feet of sea water) with 20-min bottom times, oxygen decompression at 20 and 10 fsw, and a daily 6-h surface interval. The tables were computed by a statistical procedure and had an estimated DCS risk of less than 0.01% (19). These

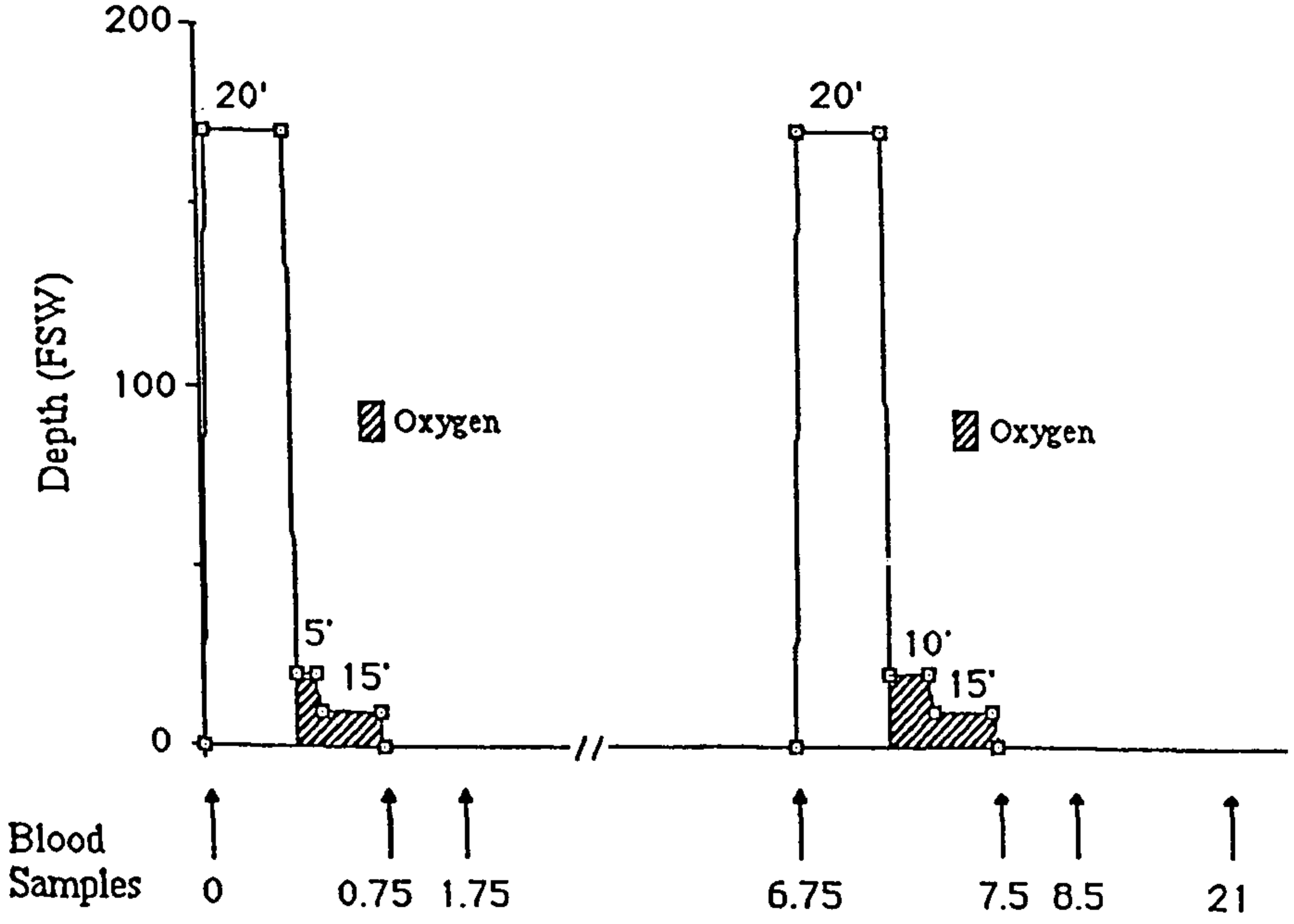

Fig. 2. Dive profile and blood sampling times. Dive depths were 170 fsw with 20-min bottom times, oxygen decompression at 20 and 10 fsw, and a 6-h surface interval. Estimated DCS risk <0.01%.

tables have been used in over 6000 air dives and have resulted in only 3 cases of pain-only DCS, a 0.05% incidence.

Subjects

The volunteers were 18 healthy, qualified divers aged 22–41. Fifteen were male and 3 female. Pregnant women and women on oral contraceptives were excluded. Subjects were asked to avoid medication containing aspirin or ibuprofen for at least 2 wk before diving. Eleven subjects were exposed to a single 20-min dive to 170 fsw, and 14 subjects were exposed to 2 such dives separated by a 6-h surface interval (Fig. 2). Each dive series began at the same time of day, with blood samples taken at comparable intervals. Seven divers were subjects in both the single and repetitive dives at least 2 wk apart.

Chamber facilities

Three to five subjects were exposed per dive in a multiplace hyperbaric chamber. The chamber was pressurized with compressed air, and oxygen was supplied during decompression through tight-fitting face masks equipped with an overboard dump system for exhaled gases. The compression rate was about 80 fsw/min (fpm) which raised the chamber temperature to 130°F transiently. An environmental control system reduced the chamber temperature to 70°–80°F within 5 min. The subjects were encouraged to move about the chamber while at 170 fsw but not to exercise. Decompression from 170 to 60 fsw occurred at 60 fpm and at 30 fpm from 30 fsw to the surface. There was a brief temperature drop to 32°F during decompression.

Doppler monitoring

Doppler monitoring was performed in real time by an experienced observer according to procedures developed at the Defence and Civil Institute of Environmental Medicine (DCIEM) (20). Periodic real time review of Doppler scores was performed by three other trained observers at random intervals. Their results were in agreement. VGE signals were graded from 0 to IV by the Kisman-Masurel code. The Doppler instrument (Techno Scientific, Inc.) operated at a continuous frequency. Each subject underwent predive monitoring followed by postdive readings within 15 min of surfacing and approximately every 30 min thereafter. Subjects were monitored until there was a decreasing trend in bubble score. The precordium was monitored with the subject standing at rest and after a deep knee bend. The region over each subclavian vein was monitored at rest and after a hand squeeze (20). Signals and voice annotations were recorded on separate channels of a Marantz type system.

Blood sample analysis

Blood samples were drawn by venipuncture rather than by an indwelling catheter to avoid nonspecific activation of complement. For sensitivity testing of blood drawn predive, 3-ml samples were taken in vacutainer tubes containing 2 units of heparin per ml blood. The heparin prevented clotting but permitted complement activation during subsequent incubation with air. For establishing the time course of intravascu-

lar activation during surface intervals and postdive periods, sequential samples 3 ml in volume were drawn in standard EDTA-containing vacutainers. The EDTA chelated calcium and magnesium, thus blocking further complement activation. Samples were taken according to the following schedule. The time of blood sampling in the single dive subjects was the same as that in the first dive of the repetitive dives:

1. Predive 1 (0 h)
2. Immediately after dive 1 (0.75 h)
3. One hour after dive 1 (1.75 h)
4. Immediately before dive 2 (6 h after dive 1; 6.75 h)
5. Immediately after dive 2 (7 h after dive 1; 7.5 h)
6. One hour after dive 2 (8 h after dive 1; 8.5 h)
7. *Twelve to 14 h after dive 2 if it was made (21 h).*

The assay for complement activation involved measuring the number of C3d fragments per red cell using a radioimmunoassay. Red cells were washed 3 times in veronal-buffered isotonic saline (VBS) to remove C3d fragments in the fluid phase. The pellet was resuspended in VBS at a concentration of 2–5 $\times$ 10^8 cells $\cdot$ ml^{-1}. The remaining RBC-bound C3d antigen (expressed on C3d or C3d-containing fragments) was measured by binding mouse monoclonal antihuman C3d antibody labeled with ^{125}I using chloramine-T. Duplicate samples containing 50 μl cell suspension and 50 μl radiolabeled C3d antibody (Cytotech, San Diego, CA) at 2 ng $\cdot$ μl^{-1} were incubated over a 3:2 mixture of *n*-butyl and bis (2-ethylhexyl) phthalate oils for 20 min at room temperature. Subsequently, the samples were centrifuged through the oil to separate cell-bound C3d antibody from unbound C3d antibody. The radioactivity of cell-bound C3d antibody in the tube tips was counted, and the amount of C3d antibody bound per cell was calculated using a method reported previously (17). The results were expressed as 10^{-12} μg anti-C3d antibodies per red cell. Of note, 2% of total anti-C3d binding of antibodies can be attributed to leukocytes because cell washings and the phthalate oils only exclude platelets. The value of the response-error relationship was less than 0.04.

Sensitivity to complement activation by bubbles was determined in predive blood samples after dividing them into 3 aliquots in 250-μl polypropylene tubes. One tube (the blood control) contained blood alone. A second tube (the blood-bead control) contained blood and a siliconized steel bead. A third tube (the air-challenge sample) contained blood, a bead, and a 30-μl air bubble. The tubes were rotated at 18 rpm for 1 h at 37°C. During rotation, the siliconized bead increased the air-blood surface area by breaking the bubble into about six to eight smaller bubbles. Attempts at bubbling air through the blood were unsuccessful because surface tension caused blood to foam out of the tube. Small air and blood volumes were used to minimize blood shear but maintain a reasonably large air:blood ratio. After rotation, complement activation was stopped by placing the sample tubes in ice, and the C3d assay was performed.

RESULTS

The results of the in vitro sensitivity study are presented in Fig. 3. Figure 3*A* shows the distribution of RBC-bound C3d in the subject population for the blood controls.

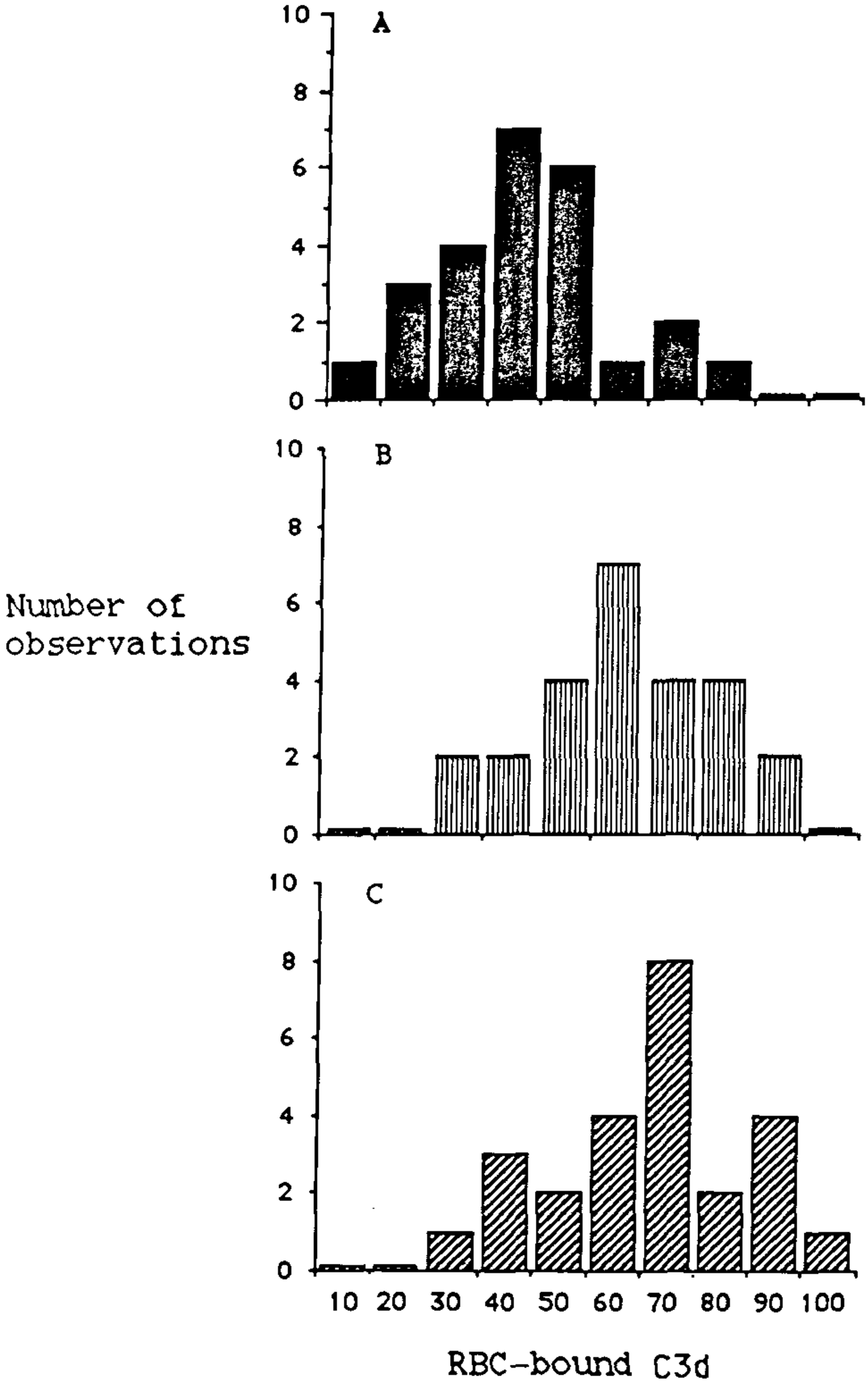

Fig. 3. Distribution of RBC-bound C3d (10^{-12} μg of anti-C3d antibodies/RBC) in the subject population during in vitro sensitivity testing; *A*, blood controls; *B*, blood and bead controls; *C*, challenge by bubble and bead. Data were generated from 25 observations (11 single dives + 14 repetitive dives) on 18 subjects.

Figure 3*B* shows the distribution for the blood-plus-bead controls and Fig. 3*C* shows the blood samples that were incubated with the bubble and bead. The rightward shift in distribution from Fig. 3*A* (blood controls) to Fig. 3*B* (bead controls) and to Fig. 3*C* (bubble plus bead challenge) indicates progressively greater complement activation. Generally, subjects with higher amounts of RBC-bound C3d in Fig. 3*A* showed higher amounts of RBC-bound C3d in Fig. 3*C*. While Fig. 3 gives the general impression of a normally distributed population with a single peak, there is a suggestion of a second population peak at the higher C3d values. There are too few subjects in the secondary peak, however, to confirm its presence. The distribution of changes in RBC-bound C3d in individual subjects was in agreement with the absolute values.

In the 2 series of dives performed by one population of divers, there were no cases of DCS. Approximately half of the subjects, however, reported itching during or after decompression from both the single and the repetitive dive. This symptom was probably not related to complement activation (*see* below and Fig. 6). It may have been a result of complement-independent platelet aggregation and subsequent release of histamine (9). Of the 14 subjects making the repetitive dive series, VGE were

detected by Doppler in all divers after the first dive, and in 12 subjects after the second dive. Ten of 11 subjects making the single dive had VGE. Figure 4*A*, *B* show the distributions of maximum bubble grade (after movement) from either subclavian or precordial regions for the single and repetitive dives. It has been demonstrated previously that the maximum bubble grade from any location is related to risk of DCS (21).

Maximum bubble scores for the single dive ranged between 0 and III with a mode of I. For the repetitive dives, the maximum scores were between I and III with a mode of II for the first dive and between 0 and IV with a mode of I for the second dive. There was a nonstatistically significant decrease in maximum Doppler score and in time to maximum score after the second dive compared with the first.

Figure 5 shows the relationship between maximum changes in RBC-bound C3d and maximum bubble grade for all dives. No correlation was found by Spearman Rank Correlation test. Subjects demonstrating grade 0 or III bubbles after a single dive failed to show complement activation. Subjects with grade I bubbles after the dives demonstrated a wide range of RBC-bound C3d levels. There was no definite trend toward an increase in complement activation for subjects with higher bubble grades.

Figure 6 shows the changes of RBC-bound C3d during the pre- and postdive periods for both single and repetitive dives. There was no significant difference in C3d binding between predive controls and postdive samples after the single dive or after the first dive of the repetitive series. Immediately after and 1 h after the second dive, however,

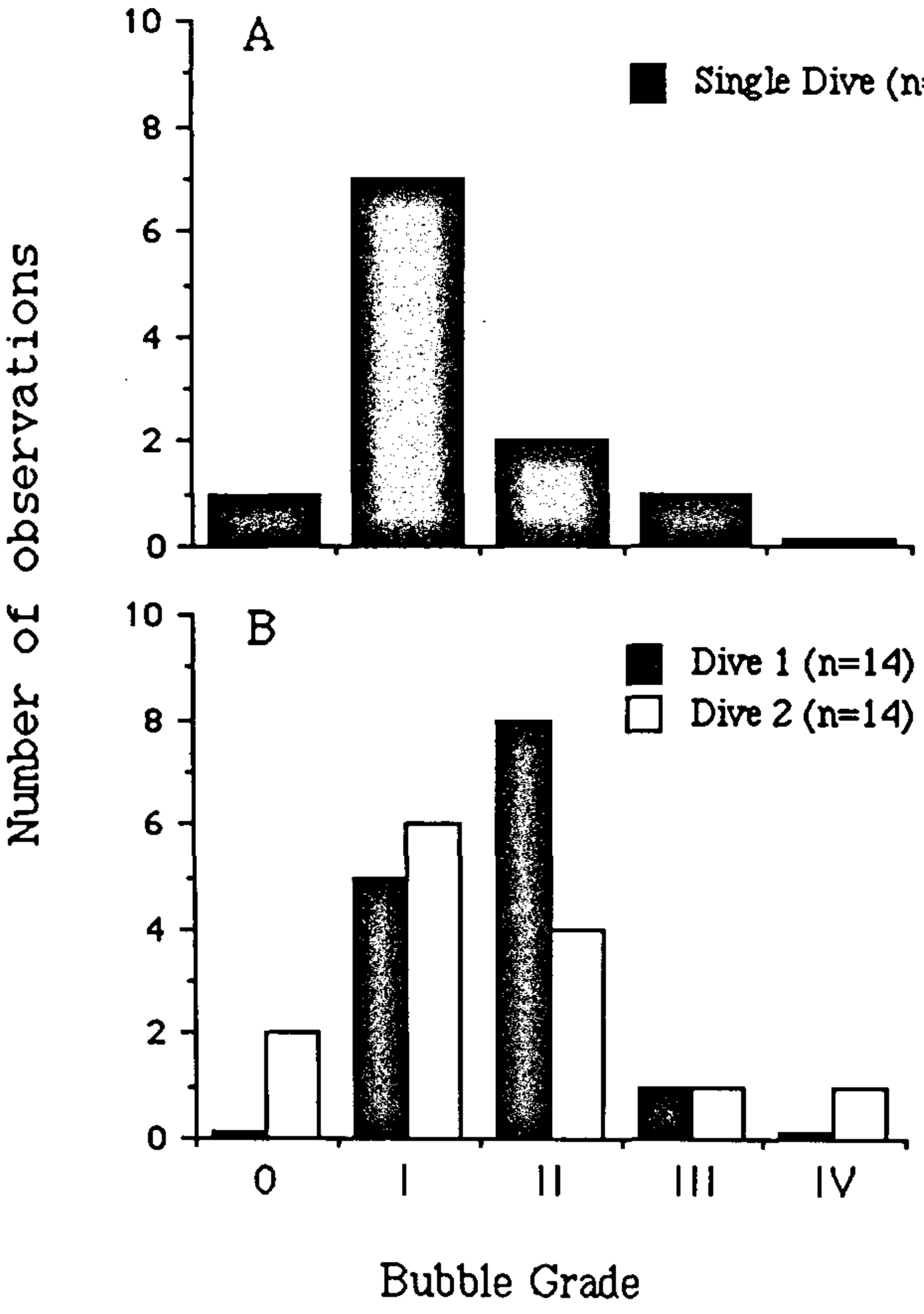

Fig. 4. Distribution of maximum bubble grades during movement from either the precordial or subclavian regions: (*A*) single dive; (*B*) repetitive dive.

Fig. 5. Relationship between maximal bubble grade during movement and maximal changes in RBC-bound C3d (10^{-12} μg of anti-C3d antibodies/RBC) after repetitive air diving. No correlation was apparent by Spearman Rank Correlation test in all dives.

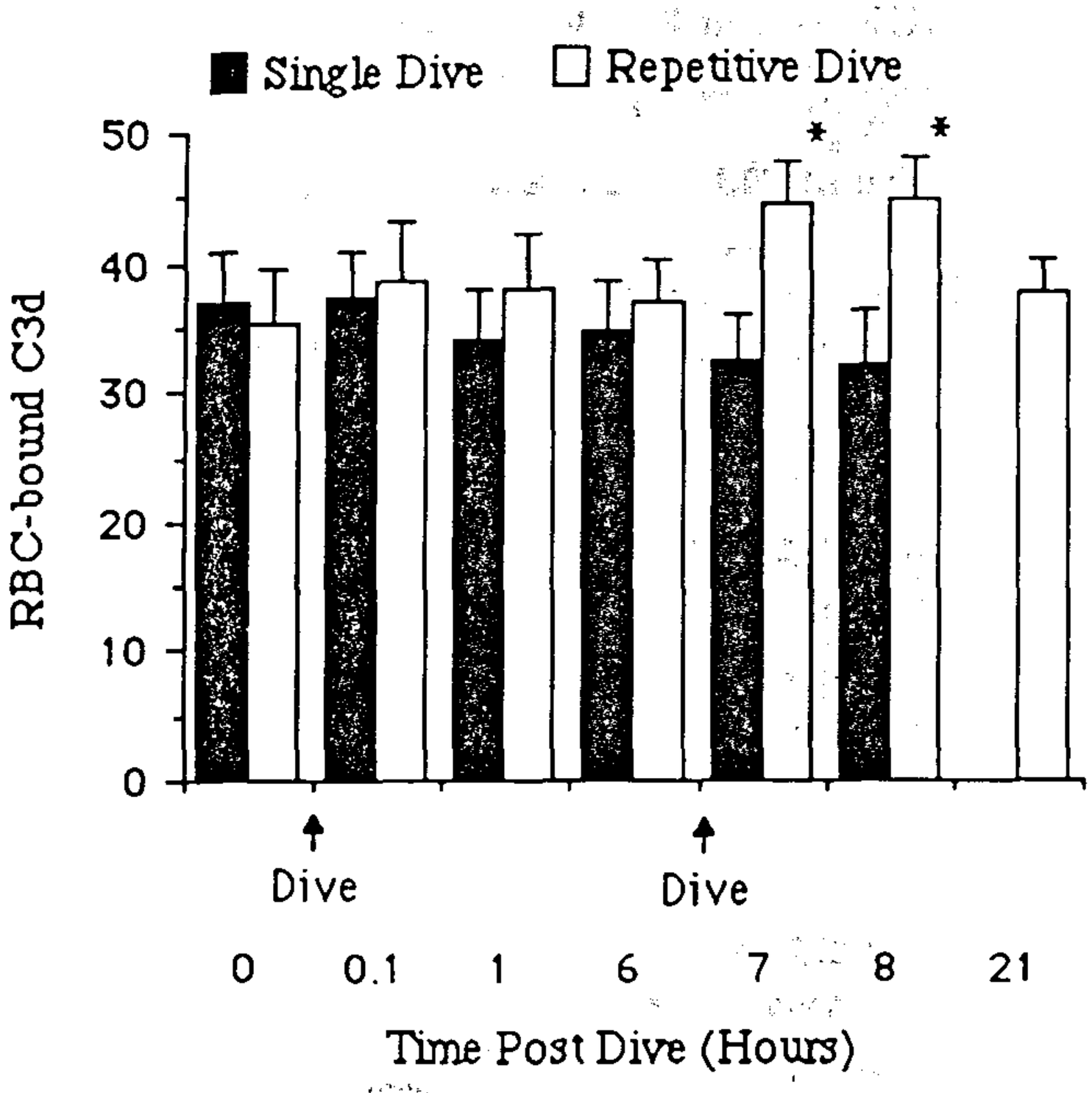

Fig. 6. Complement activation after single and repetitive dives. No significant difference was found after a single dive or after the first dive of the repetitive series. However, RBC-bound C3d (10^{-12} μg of anti-C3d antibodies/RBC) did increase immediately after the second dive, but returned to baseline by 21 h after the beginning of the experiment.

RBC-bound C3d increased by 26 and 27%, respectively ($P < 0.05$, analysis of variance). RBC-bound C3d returned to baseline by 21 h after the first dive. A comparison of the maximum change in the amount of RBC-bound C3d in the 14 subjects after the repetitive dive with the results of their in vitro predive sensitivity studies revealed no relationship by regression analysis (R-squared 0.056).

DISCUSSION

Previous studies of complement in diving have indicated that subjects could be classified into populations that were sensitive or insensitive to complement activation according to the magnitude of their C5a responses (13). This relationship held, moreover, for both control and bubble-incubated plasma samples and correlated with the

occurrence of DCS (13, 14). The RBC-bound C3d assay did not allow us to make a similar categorization, although the data suggest two populations that might be more apparent in a larger group of subjects (Fig. 3). The complement activation by the siliconized bead is consistent with earlier observations of others (22) that artificial surfaces activate complement via the alternative pathway independent of the activation by bubbles. This suggests that substances having more stable but less provocative surfaces than siliconized bead might be useful for complement sensitivity testing. Blood shear, which may have activated complement in other studies (13, 14), also may have contributed to the variability of our measurements despite efforts to maintain gentle motion of the blood.

C3d did not increase after the single dive but did increase after the repetitive dive in all subjects by a mean of 27%. Although the single dive itself was not sufficient to activate much complement, the first dive of a repetitive series seemed to potentiate C3d binding by subsequent dives. These results are consistent with the hypothesis of Ward and associates (13, 14) that complement is depleted by repetitive diving even when DCS does not occur. Therefore, increases in RBC-bound C3d could be a potential marker of decompression stress; however, more data will be needed to confirm this hypothesis. Although the increase of RBC-bound C3d after repetitive diving suggested increased decompression stress, the results of Doppler monitoring did not indicate a similar effect. Blood-bubble interaction has been proposed to explain complement activation (9, 10, 13, 14), but another mechanism seems more likely since there was no correlation between C3d and intravascular bubbles. Other factors, such as physical exertion, extended oxygen breathing, and anxiety, also may have been involved.

Since VGE correlate weakly with DCS, the lack of correlation of complement activation with VGE is not totally unexpected. The development of overt DCS may require complement activation beyond that reported here and may involve components further down the cascade or other mediators such as thromboxane and prostacyclin (23). Low grade complement activation, as we demonstrated for repetitive dives, may not be sufficient to initiate DCS, but it may lead to complement depletion and protect divers from DCS after subsequent dives (13, 14). A definitive study would measure RBC-bound C3d and C5b-9 simultaneously in vivo during repetitive dives with a nonzero incidence of DCS.

A significant reason for slow progress in understanding DCS has been the difficulty of making physiologic and biochemical measurements related to susceptibility to DCS. Although our data suggest repetitive dives resulted in increased RBC-bound C3d, the hematologic events preceding and following this increase are not understood. Improved assays of other complement components and other mediators of tissue injury may ultimately provide the necessary tools to clarify the relationship between decompression stress bubbles and DCS.

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