Human red blood cell aging at 5,050-m altitude: a role during adaptation to hypoxia

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SAMAJA, MICHELE, LAURA BRENNA, SONIA ALLIBARDI, AND PAOLO CERRETELLI. *Human red blood cell aging at 5,050-m altitude: a role during adaptation to hypoxia.* J. Appl. Physiol. 75(4): 1696–1701, 1993.—To test the hypothesis that the human red blood cell aging process participates actively in the adaptation to hypoxia, we studied some physical and biochemical hematologic variables in 10 volunteers at sea level (SL) and after 1 (1WK) or 5 wk (5WK) of exposure to 5,050-m altitude. The 2,3-diphosphoglycerate-to-hemoglobin ratio (2,3-DPG/Hb) was 0.88 ± 0.03 (mol/mol) at SL and increased to 1.08 ± 0.03 ($P = 0.002$) and 1.28 ± 0.05 ($P < 0.0001$) at 1WK and 5WK, respectively. The average red blood cell density (D_{50}), which is inversely proportional to the fraction of young red blood cells and is therefore an index of the red blood cell aging process, was 1.1053 ± 0.0007 g/ml at SL and decreased to 1.1046 ± 0.0008 g/ml (NS) and 1.1018 ± 0.0008 g/ml ($P < 0.0001$) at 1WK and 5WK, respectively. D_{50} was correlated with 2,3-DPG/Hb at SL ($P = 0.004$), only weakly at 5WK ($P = 0.1$), but not at all at 1WK. The arterial O_2 saturation was correlated with the change of 2,3-DPG/Hb in 1WK ($P = 0.02$) and that of D_{50} in 5WK ($P = 0.04$). It is concluded that short-term (1WK) increase of 2,3-DPG/Hb is not associated with the erythropoietic response but is presumably due to respiratory alkalosis. By contrast, after prolonged hypoxia (5WK), erythropoiesis may provide an efficient way for increasing blood 2,3-DPG through an augmented proportion of young red blood cells.

2,3-diphosphoglycerate; erythropoiesis

PRESERVATION OF ADEQUATE TISSUE O_2 supply is of critical importance in hypoxia. Circulatory, respiratory, and erythropoietic adjustments are primarily involved in the acclimatization process (2, 19, 31, 34). An important functional role may also be attributed to the increased affinity for O_2 of the red blood cell (RBC) by way of higher 2,3-diphosphoglycerate (2,3-DPG) concentration (13, 14). Hyperventilation-induced alkalosis (21) appears to trigger the initial increase of 2,3-DPG through stimulation of RBC phosphofructokinase (5, 8, 13). However, the discrepancy in change between blood pH, which returns to near normal values in a few days (16) [although never completely (18)], and [2,3-DPG], which remains high, suggests alternative mechanisms regulating [2,3-DPG] in sojourners and altitude natives.

The mature RBC is unable to perform certain functions that normally occur in most aerobic cells, including the synthesis of proteins and the generation of energy from oxidative processes requiring O_2. The inability to repair extensive damage, such as unbalanced metabolism or altered ion homeostasis, confers on the human RBC the unique characteristics of a limited 120-day life span, as assessed by ^{59}Fe tagging (1, 6) and carbon monoxide rebreathing (29) techniques. This imposes the set point between the production of new RBCs by the bone marrow and their removal from the circulation by the reticuloendothelial system. The latter process occurs at random in some animal species but is selective for aging RBCs in humans (4). Old RBCs are characterized by reduced deformability (15), altered intracellular Ca^{2+} handling (25), and energy depletion (3, 12, 28). Two important features of these alterations are *1*) the progressive inactivation of the membrane Na^+-K^+-adenosinetriphosphatase, leading to cell shrinking without alteration of its content, and *2*) the progressive reduction of the RBC concentration of 2,3-DPG during RBC aging, with associated increase of the RBC-O_2 affinity (10, 24, 27). Although the former feature allows an estimation of the RBC age from its density, the latter provides an attractive explanation for the high [2,3-DPG] found at altitude both in sojourners and natives. Indeed, it may be hypothesized that the stimulation of erythropoiesis by hypoxia also increases the fraction of young RBCs characterized by high 2,3-DPG levels, but so far little experimental evidence supports this hypothesis.

RBC aging was recently correlated with O_2 affinity in some subgroups of a healthy human population. It was shown that the high [2,3-DPG] needed to compensate some anemic situations was conveniently met by lowering of the average age of the circulating RBCs (24). Thus, the RBC aging process actively participates in the adaptation to anemia (23).

The aim of the present investigation was to test the hypothesis that the RBC turnover is relevant also in response to chronic hypoxia. For this purpose, we assessed RBC aging from measurement of density and altitude-related changes of several hematologic variables in a group of volunteers during altitude acclimatization. In addition, we compared these data with those obtained in a group of local altitude natives. Possible cause-effect relationships between the stimulus (hypoxia) and the responses (increase of 2,3-DPG, decrease of average RBC density) were investigated, correlating the changes of [2,3-DPG] and RBC age with the O_2 saturation of arterial blood ($\%Sa_{O_2}$).

MATERIALS AND METHODS

Subjects. A first group of 10 Caucasian volunteers (8 males and 2 females) was studied at sea level (SL) 2 mo before departure and after 1 (1WK) and 5 wk (5WK) of exposure to 5,050 m (average barometric pressure 420 Torr). All subjects [age 35.5 ± 3.4 (SE) yr] were normally living at altitudes <300 m and had no symptoms of metabolic or blood disorders. The subjects arrived at the Ev-K2 Pyramid laboratory located at 5,050 m near the base camp of Mt. Everest (Nepal) after 1 wk of trekking at altitudes ranging from 2,600 to 5,050 m to allow proper acclimatization. Apart from some discomfort in the days after arrival at 5,050 m, none of the subjects experienced symptoms of mountain sickness. Unlimited palatable caloric and water intake decreased the body weight loss to 0.7% (NS) and 4.6% ($P < 0.05$) after 1 and 5 wk at altitude, respectively (20). The physical and mental activities after reaching the laboratory were comparable to those of normal life.

A second group of local male natives (NAT, $n = 10$, age 24.1 ± 1.8 yr), dwelling at altitudes ranging from 1,300 to 2,800 m but occasionally residing at 5,050 m for 1–3 wk, also participated in the study.

Blood samples. Venous blood (3 ml) was withdrawn at rest from an antecubital vein into heparinized syringes or vacutainers and immediately chilled in an ice bath. Some of the analyses required immediate processing, and most of the operations described below were performed within 10 min after withdrawal unless otherwise stated.

RBC density profiles. The technique employed to determine the RBC density profiles of the samples was described previously (24). Briefly, a stock hyperosmotic solution containing 2.66 M NaCl and 0.09 M KCl was prepared. Two isotonic solutions were obtained daily, mixing 5.5 vol of the hyperosmotic solution with either 65 or 88 vol of Percoll (Sigma Chemical, St. Louis, MO) and balancing to 100 vol with deionized water to yield densities of 1.090 and 1.120 g/ml, respectively. Intermediate densities were obtained, mixing in appropriate ratios the above two solutions. The density gradient profiles were obtained using 10 solutions with increasing density in 0.003-g/ml steps.

Small centrifuge tubes (4 mm ID × 39 mm long) were loaded with 0.15 ml of the isotonic solution with the desired density. Fresh blood (0.05 ml) was carefully layered over them. The tubes were immediately centrifuged at 12,000 rpm for 2 min. After centrifugation, the blood layers above and below the Percoll were recovered by a syringe and diluted separately in accurately dispensed 5 ml Drabkin's reagent (nominal accuracy ±0.01 ml). The two solutions were labeled "top" and "bottom," respectively. Their optical absorbance (A) was measured in a 1-cm path length cuvette at 540 nm after 60–120 min of incubation at room temperature. The ratio $A_{top}/(A_{top}+A_{bottom})$ yields the fraction of RBC lighter than the actual density of the Percoll solution. This value was used to build the density vs. light RBC curves shown in Fig. 1.

Perchloric acid extracts. Immediately (≤1 min) after withdrawal, 0.25 ml blood was extracted with 0.6 ml of 1 M perchloric acid, chilled in an ice bath for 5 min, and spun at 12,000 rpm for 3 min. Four hundred microliters of the supernatant were neutralized with 0.1 ml of neutralizing solution containing 0.5 M KOH and 1 M KH_2PO_4, pH 7, and were frozen to −30°C. In this form, the samples were shipped to the laboratory in Milan for high-performance liquid chromatographic analysis of ATP. Preliminary experiments (not shown) indicated that the extracts neutralized as described above remained stable for ≥2 mo. The high-performance liquid chromatographic equipment (Kontron Instruments, Milan, Italy) was composed of two pumps (model 420) and a detector (model 432, UV/Vis) set at 210 nm. The 3-μm Supelcosil LC18 column (Supelco, Bellefonte, PA) was equilibrated with 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium sulfate, and the sample (20 μl) was injected and eluted using a composed gradient with a buffer containing 0.1 M KH_2PO_4, 4 mM tetrabutylammonium sulfate, and 90% (vol/vol) CH_3CN. The analysis was completed in 25 min, and data were analyzed with the Kontron's dedicated software against a blank.

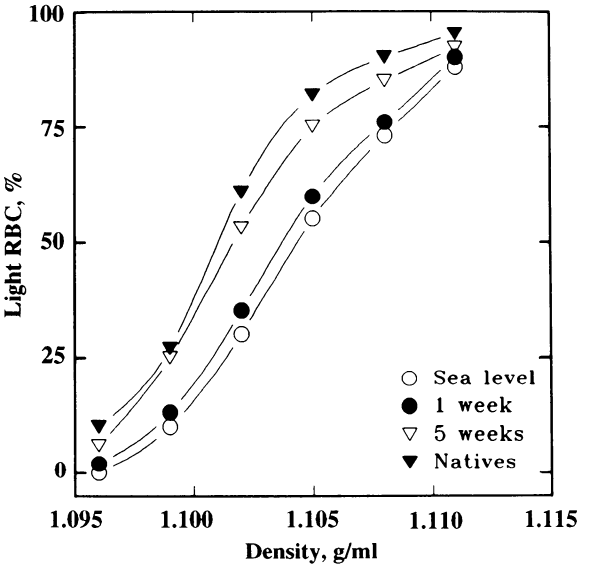

FIG. 1. Red blood cell (RBC) density profiles in 4 groups under study. Each curve is representative of 10 different subjs. Error bars are omitted for clarity.

An additional acid extract was prepared on fresh blood to measure [2,3-DPG] with the kits supplied by Boehringer Biochemia (Mannheim, Germany) within 2 h of withdrawal. 2,3-DPG is expressed as the molar ratio of [2,3-DPG] to hemoglobin concentration ([Hb]).

Additional measurements. Percent Sa_{O_2} was measured at rest by ear oximetry (Biox 3740 pulse oximeter, Ohmeda). Blood [Hb] was determined with the standard Drabkin method of reading the absorbance at 540 nm (Spectronic 301, Milton Roy, Rochester, NY) and was standardized by calibrating the diluent volumes with a balance. Erythropoietin (EPO) was assayed in Milan on frozen serum samples with a standard laboratory assay kit (Milenia, Medical Systems, Genoa, Italy).

Calculations and statistical tests. The RBC density gradient profiles were expressed quantitatively by a single parameter interpolating the density at which the light RBC fraction is 50% of the total (D_{50}). For this purpose, least-square fitting to empirical functions (TableCurve

TABLE 1. *Hematologic variables in 10 subjs at sea level and after 1 and 5 wk of exposure to altitude*

	[Hb], g/l			[2,3-DPG]/[Hb], mol/mol			[ATP]/[Hb], mol/mol			D_{50}, g/ml			EPO, mU/ml			Sa_{O_2}, %		
Subject	SL	1WK	5WK	SL	1WK	5WK	SL	1WK	5WK	SL	1WK	5WK	SL	1WK	5WK	SL	1WK	5WK
LB	141.7	203.5	174.6	0.98	1.10	1.45	0.221	0.242	0.283	1.1031	1.1033	1.0982	17.1	17.7	17.8		83	86
FC	157.8	190.4	185.6	0.92	0.93	1.37	0.233	0.247	0.236	1.1067	1.1013	1.1024	11.8	54.0	62.0		83	79
AC	157.8	214.9	197.7	0.96	1.05	1.30	0.187	0.188	0.224	1.1043	1.1017	1.0990	11.9	13.0	17.0		85	80
FE	156.2	206.8	195.9	0.78	1.10	1.13	0.217	0.216	0.261	1.1067	1.1056	1.1024	14.3	67.0	25.0		82	81
BG	162.6	227.3	212.3	0.92	1.02	1.10	0.168	0.215	0.252	1.1047	1.1061	1.1038	14.6	38.0	33.0		90	93
BK	156.2	179.4	183.4	0.74	1.27	1.32	0.261	0.204	0.233	1.1085	1.1048	1.1053	14.2	20.0	16.0		77	81
SM	143.3	196.2	182.7	1.04	1.18	1.58	0.187	0.204	0.324	1.1014	1.1013	1.0997	5.0	14.2	13.5		83	84
MN	156.2	209.8	204.3	0.72	0.95	1.08	0.269	0.265	0.268	1.1073	1.1076	1.1047	15.9	34.0	25.0		82	83
AR	169.1	215.3	205.7	0.81	1.16	1.11	0.191	0.230	0.249	1.1044	1.1075	1.1007					74	82
HS	150.5	190.0	181.9	0.92	1.03	1.34	0.190	0.141	0.251	1.1058	1.1064	1.1018					82	84
Mean	155.1	203.4	192.4	0.88	1.08	1.28	0.212	0.215	0.258	1.1053	1.1046	1.1018	13.1	32.2	26.2		82	83
± SE	± 2.6	± 4.6	± 3.9	± 0.03	± 0.03	± 0.05	± 0.011	± 0.011	± 0.009	± 0.0007	± 0.0008	± 0.0008	± 1.3	± 7.0	± 5.6		± 1	± 1
P		<0.0001	<0.0001		0.002	<0.0001		NS	0.01		NS	<0.0001		0.02	0.008			

Values are raw data and means ± SE of Caucasian subjs at sea level (SL) and after 1 (1WK) and 5 wk (5WK) of exposure to 5,050 m. [Hb], hemoglobin concn; [2,3-DPG], 2,3-diphosphoglycerate concn; D_{50}, average RBC density; EPO, erythropoietin; Sa_{O_2}, arterial O_2 saturation; *P*, significance of differences from SL (by Wilcoxon signed-rank test). EPO data for 2 subjs and Sa_{O_2} data at SL were not obtained.

software, Jandel Scientific, Corte Madera, CA) was used to determine D_{50} for each subject from the density vs. light RBC plots. To compare data obtained from the same subjects at various times, the nonparametric Wilcoxon signed-rank test was used. To compare two different populations, the nonparametric Mann-Whitney test for unpaired samples was used. The significance level was set at $P = 0.05$ (2-tailed).

RESULTS

Figure 1 shows typical RBC density gradient curves for the two groups under study at the investigated exposure conditions. Sojourning at altitude resulted in decreased RBC density. Local natives exhibited the lowest RBC density. The slopes of the density curves were essentially equal in their middle portion. Therefore, the D_{50} was a good estimate of the average RBC density and a criterion for characterizing the groups.

In Table 1, raw data of the subjects at SL before departure and after 1WK and 5WK of exposure to altitude are reported. All parameters were altered by hypoxia, but the changes of [Hb], [2,3-DPG]/[Hb], EPO, and $\%Sa_{O_2}$ occurred more rapidly than those of [ATP]/[Hb] and D_{50} (Fig. 2). It is possible that some degree of dehydration contributed to the abrupt increase of [Hb] in the 1st wk. Figure 2 also reports the values found in local natives, with the significance of the differences between them and SL or 5WK values. [ATP]/[Hb] in NAT was near that of SL. For all other parameters, the values of NAT were close to those of 5WK. In separate experiments at SL, no effects of the dietary state of the subjects were noted on the measured variables (not shown).

If young RBCs are assumed to have high [2,3-DPG],

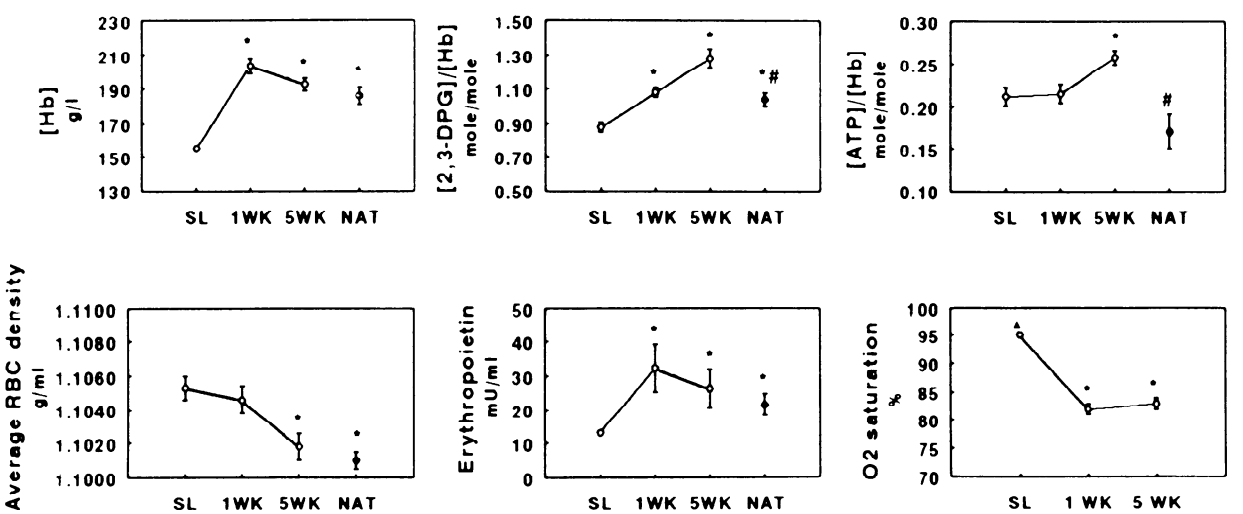

FIG. 2. Kinetics of changes of various parameters in Caucasian subjs at sea level (SL) and on exposure to 5,050 m for 1 (1WK) and 5 wk (5WK), respectively, and in local natives (NAT). [Hb], hemoglobin concn, [2,3-DPG], 2,3-diphosphoglycerate concn. ▲ Value for arterial O_2 saturation at SL was assumed and was not measured in NAT. * Significantly different from SL (Wilcoxon signed-rank and Mann-Whitney tests for unpaired samples were used for Caucasians and NAT, respectively); # significantly different from 5WK (for NAT only).

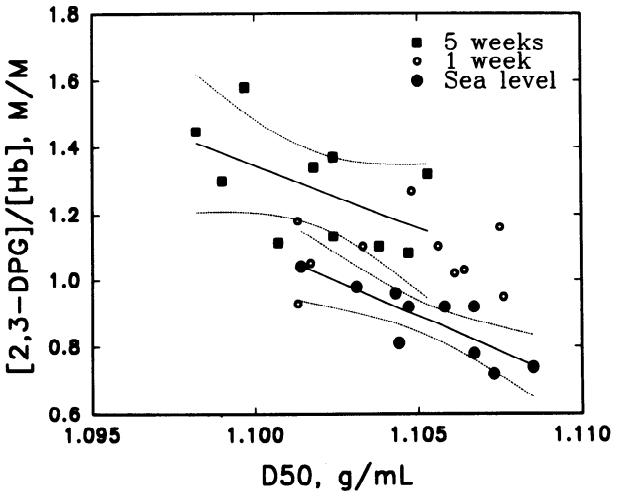

FIG. 3. Relationship between [2,3-DPG]/[Hb] ratio and average RBC density (D_{50}) at SL, 1WK, and 5WK. Statistically significant correlations were found at SL and 5WK. Best-fit lines, drawn with 95% confidence limits, are $y = -40.9x + 46.1$, $r = 0.82$, $P < 0.01$ (SL) and $y = -39.3x + 44.6$, $r = 0.55$, $P = 0.102$ (5WK). SDs for slope values were 10.3 and 21.3, respectively.

then a statistically significant correlation of [2,3-DPG]/[Hb] with D_{50} is expected. When all data obtained on Caucasians were pooled, the line of best fit was $y = -41.3x + 46.7$ ($n = 30$, $r = 0.63$, $P = 0.0002$, not shown). However, it is of greater interest to correlate D_{50} with [2,3-DPG]/[Hb] within each group, although the smaller number of data points inevitably reduces the significance of the statistical analysis. D_{50} was rather well correlated with [2,3-DPG]/[Hb] at SL ($P = 0.0045$) but not at 1WK. The correlation tended to improve in 5WK ($P = 0.102$, Fig. 3).

To test the hypothesis that hypoxia or an hypoxia-related factor is involved in the alterations of [2,3-DPG]/[Hb] and D_{50}, the size of these changes with respect to SL was correlated with the measured $\%Sa_{O_2}$ at altitude (Fig. 4). Percent Sa_{O_2} was negatively correlated with the change of [2,3-DPG]/[Hb] at 1WK ($P = 0.022$), i.e., the lower the $\%Sa_{O_2}$, the greater the change in [2,3-DPG]/[Hb]. The significant correlation of [2,3-DPG]/[Hb] with $\%Sa_{O_2}$ was lost at 5WK. On the other hand, there was no correlation of D_{50} with $\%Sa_{O_2}$ at 1WK, but a significant correlation between these two variables was detected at 5WK ($P = 0.041$). No correlation was detected between $\%Sa_{O_2}$ and [Hb], EPO, or [ATP] (not shown).

DISCUSSION

The investigated metabolic functions of the human RBC were influenced by altitude. However, the time course of the changes was different. Acute (1WK) exposure to 5,050 m primarily affected [2,3-DPG], [Hb], $\%Sa_{O_2}$, and EPO. The RBC density and [ATP], both indexes of the average RBC age (28), were influenced by prolonged exposure to hypoxia (5WK).

Decreased RBC density was associated with greater fraction of circulating young RBCs on the basis of the following assumptions (26): *1*) RBCs emerge from the bone marrow as a homogeneous population, *2*) RBCs are uniformly stressed by hypoxia, *3*) the degree of the physical stress to which the RBCs are exposed is proportional to the strength of the stressing agents, and *4*) the generated morphological changes are accompanied by proportional functional alterations. Thus, there are at least two reasons for the apparent increase of a younger RBC population: either the stressing factors become less effective, with a consequent reduction of the RBC aging rate, or the augmented erythropoiesis stimulates the production of new RBCs by the bone marrow. Although we lack direct evidence for either of the above mechanisms, we tend to exclude the former because it is not likely that a physiological stressing factor such as hypoxia decreases its effects during altitude adaptation. In contrast, the observed early increase of EPO and [Hb] after the 1st wk at 5,050 m favors the latter possibility. This hypothesis is consistent with the observed larger mobilization of iron in acute (5-day) exposure to 4,559 m (17). Normalization of EPO levels during prolonged exposure to altitude has already been reported (7, 9, 11, 30, 36).

Young RBCs are characterized by high [2,3-DPG]/[Hb] (24). Although we did not measure 2,3-DPG in density-separated RBCs, there are no reasons to believe that this relationship should fail at altitude. Therefore, the negative correlation between D_{50} and [2,3-DPG]/[Hb] in SL and 5WK values is not surprising. However, the univocal relationship between [2,3-DPG]/[Hb] and D_{50} was missing after 1 wk at 5,050 m, most likely because [2,3-DPG] was increased by factors other than decreased D_{50} or RBC aging (see below). The effect of this confounding agent(s) was progressively blunted after 5 wk at 5,050 m when the relationship between [2,3-DPG]/[Hb] and D_{50} was reestablished. However, the relatively low significance of the relationship between [2,3-DPG]/[Hb] and D_{50} in 5WK values indicates that the blunting effect was still not completely offset. In fact, in this study, a steady-state condition in the process of hypoxia adaptation was never reached. [ATP] correlated weakly with D_{50}, but

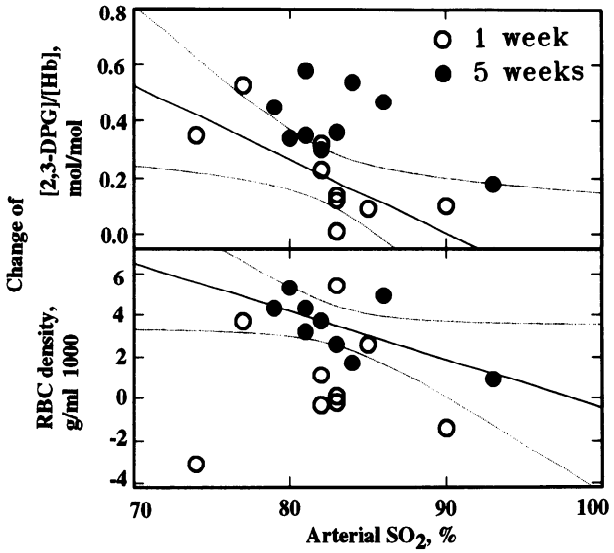

FIG. 4. Correlation of %arterial O_2 saturation ($\%Sa_{O_2}$) with [2,3-DPG]/[Hb] (*top*) or D_{50} (*bottom*) at 1WK and 5WK. [2,3-DPG]/[Hb] and $\%Sa_{O_2}$ were correlated in 1WK but not in 5WK group. However, D_{50} and $\%Sa_{O_2}$ were correlated in 5WK but not in 1WK group. Best-fit lines, shown with 95% confidence limits, are, for [2,3-DPG]/[Hb] in 1WK, $y = -2.6 \times 10^{-2}x + 2.36$, $r = 0.71$, $P = 0.022$ and, for D_{50} in 5WK, $y = 2.3 \times 10^{-4}x - 0.025$, $r = 0.65$, $P = 0.041$. SDs of slopes were 9×10^{-3} and 9.4×10^{-5}, respectively.

this feature could be expected because of the small decrease of [ATP] in the aging RBC (10).

The relationships of $\%Sa_{O_2}$ with the changes of [2,3-DPG]/[Hb] and D_{50} indicate that 1-wk altitude exposure increases [2,3-DPG]/[Hb] by mechanisms not mediated by erythropoiesis but sensitive to $\%Sa_{O_2}$, presumably hyperventilation-mediated alkalosis (21). For prolonged (5-wk) exposures, enhanced erythropoiesis should have increased the relative fraction of young RBCs, thereby raising average blood [2,3-DPG] by mechanisms independent of the body acid-base status. This stimulation appears sensitive to $\%Sa_{O_2}$, like the former, but acts on a longer time scale, of the same order of magnitude as that of the life span of the circulating RBCs (120 days) (1, 6). An alternative hypothesis for the increased [2,3-DPG]/[Hb] in hypoxia possibly involves enhanced binding of 2,3-DPG to Hb as a result of the increased fraction of deoxygenated Hb. However, serious doubt has been cast on the validity of the latter hypothesis (5).

Conclusions. Altitude exposure induces physiological and biochemical changes aimed at maintaining adequate tissue oxygenation despite reduced O_2 tension. A reduced RBC-O_2 affinity is considered useful up to 5,500 m for improving the O_2 transport without overloading the circulation, particularly at moderate work loads (23, 24). Although this response becomes disadvantageous at higher altitude or at heavy work (22), the deleterious effects of polycythemia at altitude have been well documented (32, 34, 35). In general, whereas the low sensitivity of the RBC metabolic pathways to Po_2 changes is actually a positive feature for an O_2 carrier, this does not imply that the RBC is excluded from active participation in the organism's response to hypoxia: a series of responses and feedback mechanisms are activated, leading eventually to increased [2,3-DPG]/[Hb]. These mechanisms are related both to the hypoxia-driven alkalosis and to the rejuvenation of the RBC population. This last feature would indicate that the RBC is not an inert O_2 carrier but may also have a substantial role in the process of O_2 transport during altitude acclimatization. The occurrence of such mechanisms in different geographic areas (33) and in cases of pathological hypoxia that do not directly involve Hb or the RBC (cardiac congenital disease, chronic obstruction of the lungs) is still matter for investigation.

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