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RAPID COMMUNICATION

Hemolysis Caused by Titanium Dioxide Particles

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Washed human erythrocytes were incubated with titanium dioxide (TiO_2) particles at 37°C for 1 hr and hemolysis was determined by the percentage of hemoglobin released (optical density at 540 nm; OD540) from the cells. Effects of TiO_2 on OD540 were corrected and dose-response curves were analyzed by the Hill plot. Judging from the estimated dose to cause 50% hemolysis, the anatase form of micron-scale (<5000 nm) particles was 73 and 11 times more potent than the amorphous (<50 nm) and rutile (<5000 nm) forms, respectively, whereas it was 1.3 times more potent than the nano-scale (<25 nm) anatase particles. Plasma abolished the hemolysis due to anatase and rutile forms. Thus, hemolytic effects of TiO_2 can be greatly different depending on the polymorph but not on the primary size (nano- or micron-scale) of particles. TiO_2-induced hemolysis is unlikely to occur *in vivo* because of the presence of plasma.

INTRODUCTION

Hemolysis of erythrocytes is a rapid and sensitive method to examine the effects of particles and fibers on cell membrane. Titanium dioxide (TiO_2) has often been used in such experiments as a "negative control" because it has been generally regarded as an inert substance (Wilson et al., 2000). In most cases, however, little attention has been paid to the particle form. On the other hand, with the advent of nanotechnology, ultrafine particles of TiO_2 are now widely available and this has raised concern about their possible health effects. To our knowledge, no nanoparticles of TiO_2 have been examined for their hemolytic effects.

MATERIALS AND METHODS

Anatase TiO_2 (primary size <25 nm, 99.7%) was purchased from Sigma-Aldrich Japan (Tokyo). Other forms of TiO_2 (>99.9%) and human serum proteins (albumin and γ-globulin, >95%) were supplied by Wako Pure Chemicals, Osaka, Japan. All other chemicals were reagent grade. Blood was obtained from healthy males (22 to 24 years old) into Terumo Venoject II containing heparin.

After centrifugation at 1000 g for 15 min, plasma and buffy coat were removed and the erythrocytes were suspended in isotonic buffer (30 mM Tris-HCl, pH7.4 containing 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$) of approximately equal volume. The same suspension and centrifugation were repeated two more times and packed cell volume (hematocrit) was determined. The packed cell volume to be used in the experiments was adjusted by adding an appropriate amount of the buffer (Igisu et al., 1988). All of these procedures were done at 4°C.

Titanium dioxide was suspended in the same buffer and mixed with the erythrocyte suspension. After the mixture was incubated at 37°C for 60 min with gentle shaking and an appropriate volume of the buffer was added, the mixture was centrifuged at 1000 g for 15 min and the optical density at 540 nm (OD540) of the supernatant was read using a Hitachi U-2000A spectrophotometer. Hemolysis was determined by taking OD540 of supernatant obtained after incubating with water as 100% (Igisu et al., 1988). Effects of TiO_2 on OD540 were examined by treating hemolysate in the same way as the erythrocyte suspension. To prepare hemolysate, washed erythrocytes (hematocrit

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approximately 4.5%) were frozen in dry ice-acetone and then thawed. Glutathione was measured by the method of Beutler (1975).

When necessary, the results were analyzed by Hill plot, i.e., plotting log (H/(100-H)) against log (X), where H is the hemolysis (%) and X the concentration of an agent (TiO_2, plasma, albumin or γ-globulin). Regression analysis was carried out by using Microcal Origin (version 6.0J, Microcal Software, Northampton, MA, USA) and GraphPad Prism (version 2.01, GraphPad Software, San Diego, CA, USA).

RESULTS

The anatase (< 5000 nm and <25 nm, up to 5.4 mg/ml) and rutile forms (up to 17.9 mg/ml) lowered OD540 of hemolysate by less than 5% but amorphous TiO_2 of 3.6, 5.4, and 17.9 mg/ml lowered it by 5, 9, and 46%, respectively. The dose-response curves corrected by these (Figure 1) were almost linear on Hill plot (Figure 1, inset) and the 4 lines were statistically different

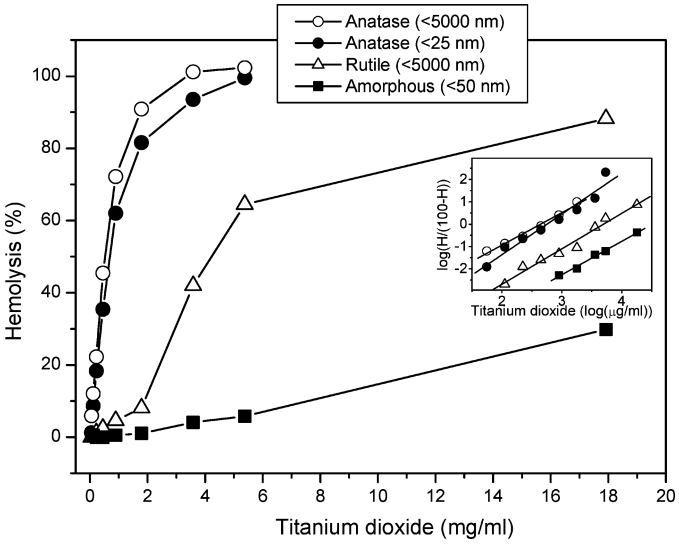

FIG. 1. Hemolysis caused by titanium dioxide of different polymorphs and primary sizes. After erythrocyte suspension (0.3 ml, hematocrit 4.5%) was mixed and incubated with buffer (0.7 ml) containing titanium dioxide or with water (0.7 ml) for 1 hour at 37°C, 1.5 ml of the buffer was added, and hemoglobin concentration (optical density at 540 nm; OD540) in the supernatant was measured to calculate hemolysis. The hemolysis was corrected by the effects of TiO_2 on OD540. On Hill plot (inset), the regression lines were; $Y = -3.848 + 1.454X$ ($R = 0.993$, $p < 0.0001$) (anatase, < 5000 nm), $Y = -5.011 + 1.821X$ ($R = 0.977$, $p < 0.0001$) (anatase, < 25 nm), $Y = -5.867 + 1.588X$ ($R = 0.987$, $p < 0.0001$) (rutile), and $Y = -6.770 + 1.501X$ ($R = 0.996$, $p = 0.0003$) (amorphous). The differences between the slopes were not significant (analysis of variance, $p = 0.261$) but those between the elevations (after fitting new regression lines to a single slope) were significant (analysis of variance, $p < 0.0001$).

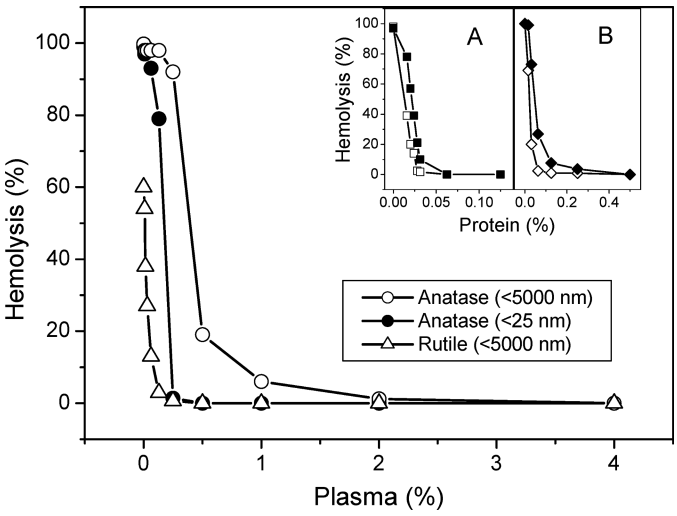

FIG. 2. Effects of plasma on hemolysis caused by titanium dioxide (5.4 mg/ml), and those of purified protein components of plasma (inset). Albumin (□) or γ-globulin (■) was added to anatase particles of primarily nano-scale (<25 nm) in A, and albumin (◇) or γ-globulin (♦) to micron-scale (<5000 nm) anatase in B. By Hill plot (not shown) analysis, the concentrations to suppress the hemolysis by 50% were estimated; 0.44, 0.12 and 0.025% of plasma against hemolysis due to anatase (<5000 nm), anatase (<25 nm), and the rutile form, respectively, 0.016% albumin and 0.021% γ-globulin in A, and 0.017% albumin and 0.055% γ-globulin in B.

(analysis of variance; $p < 0.0001$). The dose of TiO_2 (μg/ml) to cause 50% hemolysis estimated by the Hill analysis was; 444 (anatase, <5000 nm), 564 (anatase, <25 nm), 4960 (rutile) and 32414 (amorphous particles). Thus, among the particles examined, the anatase form of micron-scale was most potent to hemolyze human erythrocytes, and its potency was approximately 73 and 11 times that of amorphous and rutile forms, respectively, whereas it was 1.3 times more potent than the nano-scale anatase.

No changes were observed in erythrocyte glutathione (GSH) levels after incubating with the anatase form of titanium dioxide whereas 1 mM cupric sulfate caused clear decrease of GSH (data not shown). By adding less than 2% of plasma, hemolysis caused by anatase as well as rutile form was almost completely suppressed (Figure 2). Both albumin and γ-globulin suppressed the hemolysis caused by anatase particles (Figure 2 A, B).

DISCUSSION

The present results seem compatible with those of Sayes et al (2006) who examined effects of nano-TiO_2 particles on human dermal fibroblasts and pulmonary epithelial cells. After 48 hrs exposure to the cultured cells, they found that anatase was much more potent than the rutile form in exerting cytotoxicity estimated by "viability stain," LDH leakage, MTT assay and IL-8 production, suggesting that anatase form may impair plasma

membrane and mitochondria, and that it may stimulate production of the inflammatory mediator. By contract, our experimental system was very simple because washed human erythrocytes were used. In addition, the incubation period was much shorter (1 hr). Hence, one of the earlier and common reactions leading to the cytotoxicity of TiO_2 in different types of cells may be perturbation of the plasma membrane. However, the mechanism of membrane perturbation by TiO_2 is not clear. Since ex vivo generation of reactive species of oxygen under UV illumination correlated well with the responses of cells, Sayes et al. (2006) suggested that oxidative stress played an important part in causing the cytotoxicity. Nevertheless, we observed no changes of GSH in erythrocytes exposed to TiO_2, hence no direct evidence of intracellular oxidative stress.

In the present experiment, the hemolysis caused by anatase and rutile TiO_2 was abolished by plasma. This suggests that intravascular hemolysis is unlikely to occur. Furthermore, two major protein components of plasma, albumin and γ-globulin, suppressed the hemolysis due to anatase particles with similar potency. This is in clear contrast to the hemolysis caused by pentachlorophenol, which was completely suppressed by albumin but not at all by γ-globulin ($<0.5\%$) (Igisu, 1993). This and the difference of hemolytic potency between polymorphs suggest that physical (mechanical) factors are important in TiO_2-induced hemolysis.

Mature human erythrocytes are apparently much simpler than other cells because they are composed of only plasma membrane and cytosol without any intracellular organelles such as nuclei and mitochondria. Besides, erythrocytes can easily be obtained from humans and separated from other cells. Thus, this simple and economical method seems useful in examining particles including those of primarily nano-scale on the human cell, and the present results indicate that attention should be paid to the polymorphs of particles in such experiments.

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