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Titanium dioxide induced chemiluminescence of human polymorphonuclear leukocytes

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Summary. The ability of different titanium dioxides (TiO_2) to induce production of reactive oxygen metabolites by human polymorphonuclear leukocytes was studied. Pure rutile or anatase preparations show only a weak chemiluminescent response. Surface-modified TiO_2 causes a strong chemiluminescent response with a biphasic configuration resembling that of quartz. Sonication of the dust suspensions resulted in a strong enhancement of the chemiluminescent response, with each dust preparation showing approximately equal maximal activity. However, coated TiO_2 still exhibited a different mode of cell activation. The chemiluminescence-inducing activity of the different TiO_2 studied did not correlate with their hemolytic activity. As polyvinyl-pyridin-N-oxide (PVPNO) inhibits the chemilumescence induced by coated TiO_2 samples, it seems that both particle size and surface structure determine the mode and intensity of activation of human PMNL by TiO_2. The results point out the need for in vivo testing and comparison of different TiO_2 preparations.

Key words: Titanium dioxides – Polymorphonuclear leukocytes – Chemilumnescent response – Reactive oxygen metabolites

Introduction

Titanium dioxide (TiO_2) occurs in nature primarily in the form of the minerals rutile, anatase, brookite, and as the iron-containing mineral ilmenite ($FeTiO_3$). The major source of TiO_2 is ilmenite, and rutile and anatase pigments are mainly produced commercially. The industrial use of TiO_2 has increased and it has more or less replaced white lead and zinc white in the paint industry. The coating of rutile TiO_2 with Si, Al, Zn or organic compounds has been undertaken to improve some characteristics of the powder. Generally TiO_2 has been considered biologically inert in experimental animals [4, 10, 11, 13] and in humans [5, 15, 20, 22]. However, the question of the possible adverse effects of TiO_2 has been raised by some authors who have shown fibrosis of the lung in workers exposed to TiO_2 over a length of time [7, 14]. However, it remained unclear whether these changes were due to the TiO_2 alone or to other factors, such as coating materials used in the process of manufacturing some TiO_2 pigments.

When human polymorphonuclear leucocytes (PMNL) are exposed to particulate or non-particulate materials, the oxidative metabolism of the cells is activated, and they produce highly reactive oxygen metabolites, e.g. H_2O_2, O_2^-, $O_2°$, OH^-. These reactive oxygen species are associated with the production of light. This phenomenon, chemiluminescence (CL), can be amplified by the use of luminol [1]. The role of PMNL in asbestos- and quartz-related diseases [2, 18] and our finding that asbestos fibers and quartz stimulate CL in human PMNL suggest a role of reactive oxygen metabolites in the pathogenesis of these diseases [8].

In this study, I have chosen to compare the effect of rutile, anatase and various coated TiO_2s on human PMNL, using luminol-dependent CL. The effect of poly-vinyl-pyridin-N-oxide (PVPNO), a known inhibitor of quartz cytotoxicity and fibrogenicity, was also studied. Since it has been shown that anatase TiO_2 possesses some hemolytic activity [24], hemolysis testing of the various dusts was performed in order to investigate whether a correlation exists between hemolytic and CL activity.

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Materials and methods

Medium and chemicals. Dulbecco's phosphate buffered saline (PBS), Ca^{2+} and Mg^{2+} free PBS were obtained from Orion Diagnostica (Finland). Luminol (LKB-Wallac, Finland) was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 10^{-1} *M*, which was further diluted in Dulbecco's PBS to 10^{-4} *M* immediately prior to the experiments. Ficoll-Paque® was obtained from Pharmacia (Sweden). PVPNO was a generous gift from Dr. Helga Idel, Düsseldorf (FRG). Zymosan (Sigma Chemical Co, USA) was opsonised by incubation with normal human serum at +37°C for 30 min at a concentration of 10 mg/ml, washed three times and resuspended in Dulbecco's PBS to a final concentration of 10 mg/ml and stored until use at −20°C.

TiO_2 samples (Finntitan®, Kemira, Finland) were a gift from the manufacturer. Two samples, TiO_2-RR2 and RR, were coated rutile. RR2 was coated with Al_2O_3,SiO_2 and ZnO and organic material, for RR the organic coating was omitted. One sample TiO_2-RR-uncoated was the same material as above but without any coating. TiO_2-RU and TiO_2-AN were normal rutile and anatase samples. A sample containing 90% anatase/10% rutile was purchased from May & Baker Chemicals (England). A detailed specification of the dusts as supplied by the manufacturer is given in Table 1.

The quartz sample was fractionated Fyle quartz from Fyleverken Ltd (Sweden). The yield of particles smaller than 5 µm was 85% of w-% as determined by liquid scintillation [21].

All samples were diluted in Dulbecco's PBS to a concentration of 1 mg/ml. Prior to some of the experiments the different TiO_2 samples were heavily sonicated for 5 min.

Isolation of PMNL. Ten ml of heparinized human blood was obtained from apparently healthy blood donors. The blood was diluted in Ca^{2+} and Mg^{2+}-free PBS, and erythrocytes and granulocytes were separated from mononuclear cells by ficoll-density centrifugation [3]. Lysis of erythrocytes with NH_4Cl was then performed [23] and the resulting cells were washed twice, resuspended in PBS and counted in a Coulter counter. The cell concentration was adjusted to 5.0×10^6/ml and viability assessed by trypan blue dye exclusion. The isolated cells contained 95 to 98% granulocytes and viability was found to be > 95% in each case.

Effect of TiO_2 on cell viability. The effect of the dust samples on PMNL viability was assessed by lysozyme release and trypan blue dye exclusion. Lysozyme was measured from supernatants of human PMNs (25×10^6/ml) incubated for 15 and 30 min at +37°C with 200 µg/ml of the different TiO_2 samples (sonicated or non-sonicated). A lysoplate assay was done using human lysozyme as a standard [16]. Enzyme release was expressed as a percentage of the total release from cells repeatedly frozen and thawed.

CL assay. CL of human PMNL was measured using a LKB 1251 Luminometer (LKB) coupled to an Apple II e microcomputer. The reaction mixture (final volume 1.0 ml) consisted of 700 µl 10^{-4} *M* luminol in Dulbeccos PBS, 50 µl of the cell suspension (5.0×10^6/ml), 25 to 200 µl of the dust suspensions (1 mg/ml) or standard phagocytic stimuli, i.e. opsonized zymosan. The CL response was followed for 30 to 60 min at +37°C and the output of each sample was recorded at intervals of two minutes. In some experiments PVPNO was added to the reaction mixture (final concentration 0.1–100 µg/ml).

Hemolysis test. The red blood cell (RBC) hemolysis test was carried out as follows. Heparinized blood was washed three times in Tris-buffer (pH 7.4) by centrifugation at 3000 rpm. A 2% RBC suspension (v/v) in Tris-buffer was prepared from the RBC pellet. Two ml of the RBC suspension were mixed with different TiO_2 samples or quartz at final concentrations of 100 or 500 µg/ml and the reaction volume was adjusted to 4 ml with Tris-buffer. Controls containing only Dulbeccos PBS were included. The reaction mixtures were incubated at +37°C for 3 h and then centrifuged at 3000 rpm. The amount of liberated hemoglobin was estimated spectrophotometrically at 415 nm from the supernatants. The results were expressed as per cent of complete hemolysis achieved by treating the RBC suspension with 10 µl of Triton X 100.

Light- and electron microscopy. For light- and electron microscopy, TiO_2 suspensions (1 mg/ml) were filtered on a membrane filter (Nucleopore®, pore size 0.2 µm). The samples were gold coated and studied by with a JEOL-100 CX electron microscope.

Results

The different (sonicated or non-sonicated) TiO_2 samples did not affect cell viability as estimated by lysozyme release or trypan blue exclusion (results not shown).

A typical experiment demonstrating the CL response of opsonized zymosan (500 µg/ml), quartz (50 µg/ml) and of the different TiO_2 samples (50 µg/ml) is shown in Fig. 1. The CL output of the coated TiO_2 resembles that of quartz. The CL response of the other TiO_2 was very low at this concentration.

Table 1. Characteristics of titanium dioxide dusts

Dust sample	TiO_2 %	Al_2O_3 %	SiO_2 %	ZnO %	C(org) %	Fe (ppm)	Nano-Sizer[a] nm
RR-2	94.1	2.15	1.12	0.90	0.26	36	345
RR	94.8	2.01	1.08	0.91	0.052	42	431
RR-uncoat.	99.0	< 0.01	< 0.05	0.9	< 0.01	25	1000
AN	98.5	< 0.05	0.05	0.005	< 0.01	21	542
RU	98.7	0.04	0.19	0.28	0.010	37	328
May & Baker	not specified						

[a] Particle size was measured with a Coulter Nano-Sizer analysator using sonicated dust samples

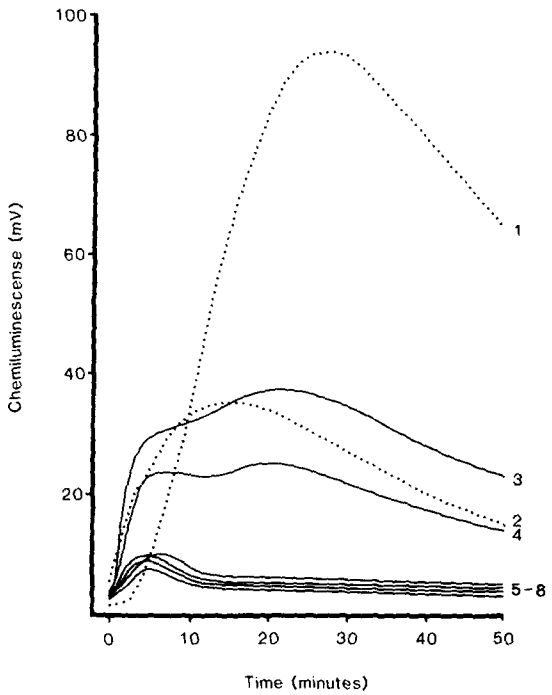

Fig. 1. A typical experiment showing the chemiluminescent output of opsonized zymosan (500 µg/ml), quartz and the different dusts (50 µg/ml). 1 = Opsonized zymosan, 2 = Fyle quartz, 3 = TiO_2-RR2, 4 = TiO_2-RR, 5 = TiO_2-RR-uncoated, 6–8 = AN, RU and TiO_2-Baker

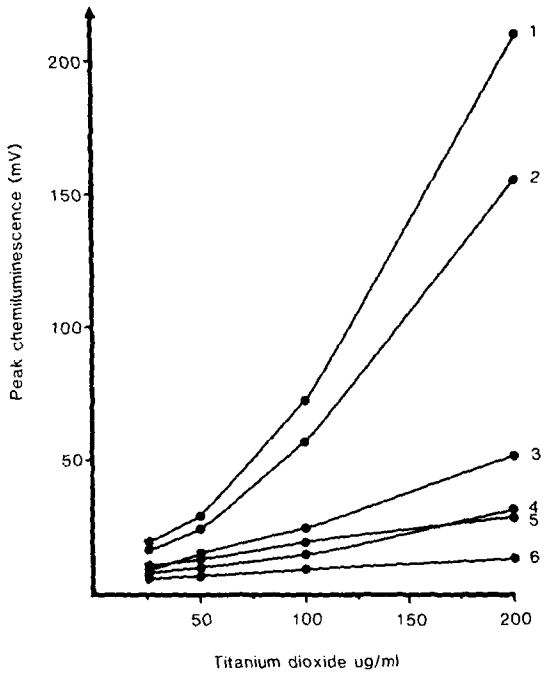

Fig. 2. Dose dependency of TiO_2-induced chemiluminescence. 1 = TiO_2-RR2, 2 = TiO_2-RR, 3 = TiO_2-AN, 4 = TiO_2-Baker, 5 = TiO_2-RR-uncoated, and 6 = TiO_2-RU. Each dot represents the mean of three different experiments

There was individual variation in the intensity of the CL response to TiO_2 and other stimulants. However, in repeated experiments the different TiO_2 dusts elicited CL responses in a consistent order of intensity. This is demonstrated in Fig. 2, which also shows the dose dependency of the response. It is evident that the coated TiO_2 exhibits the strongest CL response, especially at higher concentrations, whereas the CL output of pure rutile (TiO_2-RU) is very weak.

We have previously shown that quartz exhibits a biphasic CL response (as demonstrated by the "shoulder" at 4 to 6 min on the quartz response in Fig. 1). In experiments with TiO_2 the same observation was made concerning the surface-modified, coated TiO_2 samples. The coated TiO_2 samples showed a first peak at 4 to 8 min and a second one at 24 to 30 min, whereas the other dust samples studied showed only one peak, corresponding to the first peak of the coated samples.

The TiO_2 varied in suspendibility, as some tended to form aggregates in aqueous solution. The different samples were therefore sonicated and the effect of this procedure on the CL induction is shown in Table 2. Sonication of the TiO_2 dramatically increased their CL response. The sonication process also resulted in about the same CL peak responses for all TiO_2 dusts. However, the coated TiO_2 samples still exhibited a biphasic CL response.

Figure 3 shows light-(3a–b) and electronmicroscopic (3c–d) appearance of non-sonicated and sonicated rutile TiO_2. Non-sonicated rutile forms large aggregates, whereas these aggregates are dispersed after sonication. By electron microscopy no changes induced by sonication on the size and shape of TiO_2 can be observed.

The hemolytic activity of the different TiO_2 samples and quartz was also tested. At a dust concentration of 100 µg/ml, only quartz (used as a positive control) induced measurable hemolysis (23% of total hemolysis). At 500 µg/ml quartz induced 73% hemolysis, whereas only anatase TiO_2 exhibited weak hemolytic activity (4–5% hemolysis). This weak activity was slightly amplified by sonicating the dust samples prior to testing (9–16% hemolysis). Rutile TiO_2

Table 2. Effect of sonicated on peak CL intensity (mV) of various titanium dioxide dusts (100 µg/ml). Mean ± SD

Dust sample	Non-sonicated ($N = 6$)	Sonicated ($N = 3$)
RR-2	51.2 ± 38.2	118.3 ± 45.8
RR	42.4 ± 29.1	126.2 ± 51.9
RR-uncoated	15.8 ± 7.8	126.1 ± 58.1
AN	16.7 ± 11.2	138.6 ± 54.5
RU	8.3 ± 1.9	129.0 ± 48.2
May & Baker	11.1 ± 5.0	79.1 ± 22.8

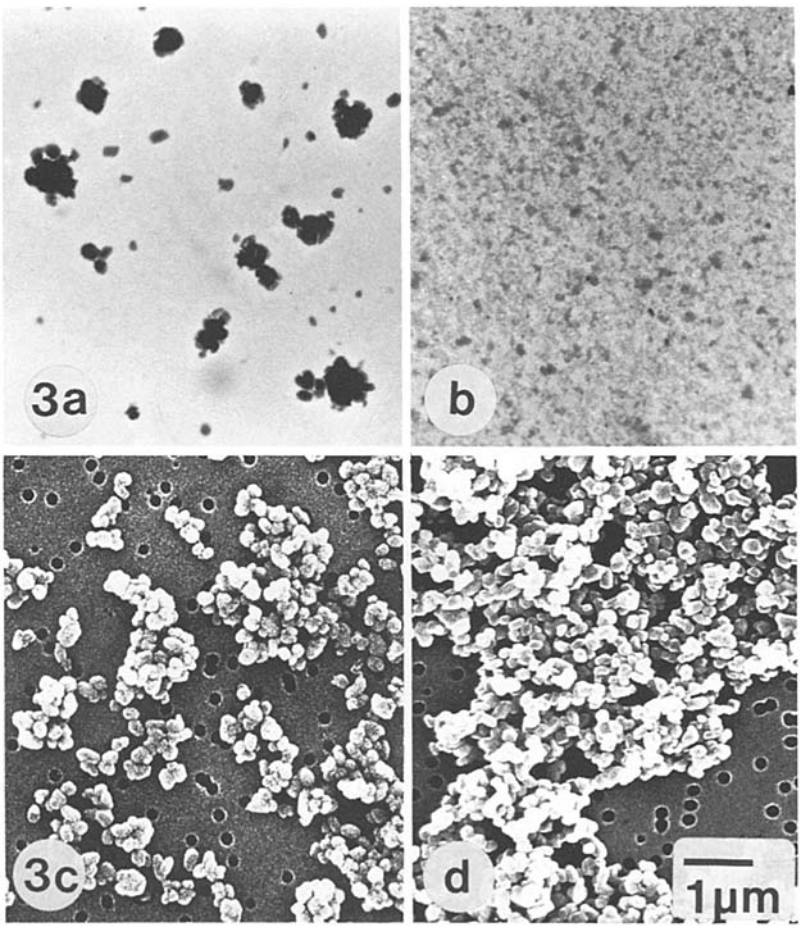

Fig. 3. Light microscopy of non-sonicated **a**, and sonicated **b** TiO_2. Original magnification 400 ×. Scanning electron microscopy of non-sonicated **c**, and sonicated **d** of the same TiO_2 samples. Magnification 10000 ×

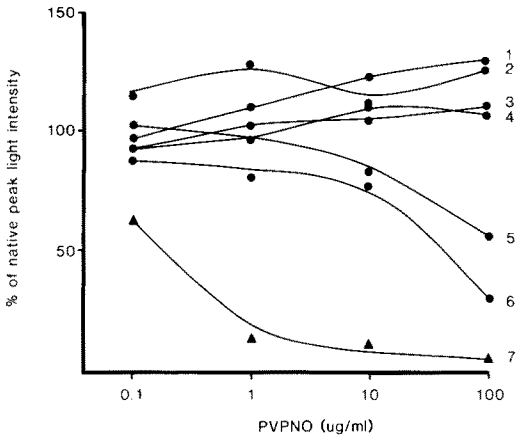

Fig. 4. Effect of PVPNO on TiO_2-induced peak chemiluminescence. 1 = TiO_2-RR-uncoated, 2 = TiO_2-RU, 3 = TiO_2-Baker, 4 = TiO_2-AN, 5 = TiO_2-RR2 and 6 = TiO_2-RR

(both coated and uncoated or sonicated and non-sonicated) showed no hemolytic activity at these concentrations.

We have previously shown that quartz stimulates the CL of human PMNL and that this stimulation can be inhibited by the use of PVPNO. Since silica or silicon compounds are used in the coating of rutile TiO_2, the effect of PVPNO on the TiO_2-induced CL was studied. Figure 4 shows the effect of PVPNO (0.1–100 µg/ml) on peak CL of sonicated TiO_2 samples and fyle quartz. High concentrations of PVPNO inhibited the response of the coated TiO_2 samples, whereas uncoated samples were unaffected or even slightly stimulated by PVPNO. When non-sonicated TiO_2 samples were examined, the inhibitory effect of PVPNO on coated TiO_2 samples was found to be less marked and a stimulatory effect on the CL response of the uncoated samples was observed (data not shown). The nature of this slight stimulatory effect of PVPNO on uncoated TiO_2 is not known.

Discussion

TiO_2 is regarded as a nuisance dust by most investigators. It possesses low cytotoxicity and is often used as a low-toxicity control dust in cytotoxicity testing [17]. The pulmonary response of rats exposed by inhalation to ilmenite [13] or pure TiO_2 [4] showed retention of TiO_2 in the lungs but very little fibrosis.

Exposure of rats to excessive amounts of rutile TiO_2 for two years caused overloading of lung clearance mechanisms. This heavy exposure by inhalation also resulted in a foamy macrophage response, alveolar proteinosis, cholesterol granulomas, etc., and even the development of cystic keratinizing squamous carcinomas was seen. However, little lung fibrosis was observed and the relevance of the development of the lung tumors was questioned by the authors [10, 11].

The effects of TiO_2 on man have not been extensively studied. In an investigation into the health of workers in an ilmenite extracting plant, no signs of occupational lung disease were observed [22]. In another study irritation of the the upper and lower respiratory tract and some functional abnormalities of the lung were observed in workers involved in the production of TiO_2 from ilmenite ore by a sulphate process. No signs of serious occupational lung disease were observed [5].

In lung tissue samples of workers heavily exposed to TiO_2, retention of TiO_2 in the lung tissue and in macrophages has been shown [7, 14, 15, 20]. Some authors found no signs of pulmonary fibrosis and indeed claimed that rutile TiO_2 is biologically inert [15, 20]. However, slight fibrosis of the lung in heavily exposed workers has been described [7]. Traces of silica and aluminium besides TiO_2 in alveolar macrophages of workers exposed to TiO_2 has led to speculation that the slight adverse effect of TiO_2 could be mediated by coating compounds such as silica or silicon [14].

In the present study, the CL activity of human PMNL stimulated with different TiO_2 samples, quartz and opsonized zymosan was compared. Surface modification of TiO_2 with silica or silicon compounds, Al or Zn greatly enhances the production of potentially inflammatory oxygen metabolites by PMNL as measured by CL. Addition of an organic coating did not modify the effect of TiO_2 on PMNL any further. Coating of TiO_2 also resulted in a biphasic chemiluminescent response which was not observed when studying uncoated samples of TiO_2.

The solubility and dispersibility of the TiO_2 samples varied considerably. Sonication of the (TiO_2) samples was performed in order to get a better aqueous solution. This resulted in a drastically higher CL output of all TiO_2 samples studied, and no big differences between different samples in peak CL were observed. However, the coated TiO_2 samples still exhibited a typical biphasic CL pattern. This suggests that particle or aggregate size and/or surface characteristics are of major importance in the TiO_2-induced production of reactive oxygen metabolites. The biphasic chemiluminescent response of the coated TiO_2 samples was evident despite sonication, suggesting a different mode of activation of human PMNL.

Anatase TiO_2 (concentration up to 20 mg/ml) has been reported to display hemolytic activity, whereas coated or uncoated rutile TiO_2 is hemolytically inactive [24]. In the present study, much lower concentrations of TiO_2 (100–500 µg/ml) were used. Using these concentrations, anatase TiO_2 seems to possess a weak hemolytic activity, which can be slightly amplified by sonicating the dust sample. Rutile TiO_2, coated or uncoated, has no hemolytic activity at the concentrations studied. Most authors have used the hemolysis test as a model for studying the nature of interaction between dust particles (especially quartz and asbestos) and biological membranes. It correlates rather poorly with fibrogenic potential [6]. There was no correlation between the weak hemolytic effect of different TiO_2 dusts and their capacity to induce CL in PMNL.

PVPNO reduces quartz-induced hemolysis, cytocoxicity and CL production by human PMN ([9] unpublished results). It has a therapeutic effect in experimental silicosis and has a therapeutic potency in man as well [19]. In this study, PVPNO was shown to inhibit the CL induced by the surface-modified coated TiO_2 samples, whereas the effect on the non-coated samples was even slightly stimulatory. However, considerably higher concentrations of PVPNO were needed for this inhibitory action than for inhibition of the quartz response. The effect of PVPNO further suggests a quartz-like action of the surface-modified TiO_2 samples.

In conclusion, TiO_2 stimulates the CL activity of human PMNL in a dose-dependent fashion. Particle size and especially surface structure of the dust determines the intensity and mode of activation. No conclusions can be drawn about the potential adverse effects of surface modified TiO_2 on the basis of these results. However, it would be of interest to compare the effects of different types of TiO_2 in vivo.

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