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Effects of Titanium Dioxide Pigments on Mouse Peritoneal Macrophages *in vivo*

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The peritoneum is well isolated from the outer world. Thus it has a defense system of its own to keep the cavity clean of different kinds of materials. The peritoneal clearing system is not as effective as that of the lung due to more random exposure of the peritoneal cavity to micro-organisms and other irritants (e.g. MIMS 1964). The peritoneal cells, mainly phagocytes, have a different pattern of hydrolytic enzymes compared to alveolar macrophages. The peritoneal phagocytes are principally glycolytic cells whereas the alveolar macrophages are aerobic cells (VERNON-ROBERTS 1972, PORTUGALOV et al. 1973, DRATH et al. 1976). The peritoneal cell recovery can be increased by giving a stimulating injection of, e.g., paraffin oil, Indian ink or carbon particles (e.g. MIMS 1964, WHALEY et al. 1972).

The peritoneal cavity's reactions to foreign particles have been studied, e.g., with plutonium dioxide and thorium dioxide (SANDERS & ADEE 1969), chrysotile asbestos (DAVIS & CONIAM 1973), crocidolite asbestos fibers (SETHI et al. 1974) and titanium phosphate fibers (GROSS et al. 1977). The cytotoxicity tests are nowadays mostly conducted by *in vitro* methods that seem to give more detailed and accurate results (e.g. ALLISON et al. 1966, MILLER et al. 1978, ZITTING & SKYTTÄ 1979, KANE et al. 1980). More often the cytotoxicity tests are studied with alveolar macrophages that in nature are exposed to different kinds of dusts and particles (e.g. SINGH et al. 1977, GABOR et al. 1978, AGARWAL et al. 1978, CAMNER et al. 1978, ARANYI et al. 1979).

The aim of this study was to calculate and compare the toxicity of six different titanium dioxide pigments to mouse peritoneal phagocytes *in vivo* for better understanding the pulmonary reaction mechanisms to some of these pigments. Special attention was paid to the hydrolytic activity changes in the peritoneal phagocytes, phagocytic activity and the effects on cell counts in the peritoneal cavity.

MATERIALS AND METHODS

Male NMRI-mice were used. They were kept in cages, 4 to 8 per cage. Water and food were given *ad libitum*. At the beginning of the experiments the age of the animals was 4-6 weeks.

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Titanium dioxide (TiO_2) pigments were given as a single intraperitoneal injection in 1 mL of 0.9% aqueous NaCl solution. The pigment dose was 5 mg per animal (Table 1). Controls received the same volume without the pigment.

Table 1. Chemical ingredients of the six titanium dioxide pigments used in the cytotoxicity tests.

Pigment	TiO_2	SiO_2	other compounds present
T 1	98.0%	-	
T 2	98.0%	0.4%	P_2O_5, CaO, Sb_2O_3
T 3 & T 4	83.0%	8.2%	ZnO, P_2O_5, CaO, Al_2O_3, Sb_2O_3
T 5 & T 6	93.7%	0.9%	" " " " " , C

The test pigments are produced by four manufacturers. T 1 is a product of MAY & BAKER (Dagenham, U.K.), T 4 is produced by KRONOS-TITAN GmbH (Western Germany) and T 6 by the BRITISH TITAN PRODUCTS Ltd (U.K.). All the other pigments are with license produced by KEMIRA Oy VUORIKEMIAN TEHTAAT (Pori, Finland).

The cells were lavaged from the peritoneum with 4 mL of cold sterile 0.25 M sucrose. The solution was injected into the peritoneal cavity and after a short and light manual massage the cell suspension was drawn into the syringe. The yield varied from 3 to 4 mL. The suspension was placed in a test tube and centrifuged for 5 min at 1500 rpm. The supernatant was then removed and replaced with 2 mL of 0.25 M sucrose. The cells were later homogenized in an Ultra-Turrax homogenizer for 30 s at 20,000 rpm.

The hydrolytic enzymes were analyzed using the p-nitrophenol method for acid phosphatase (EC 3.1.3.2.) and p-nitrophenyl-β-D-glucuronidase as substrate for β-glucuronidase (BARRETT 1972). The enzyme acitivity was calculated against the protein content that was assayed according to LOWRY et al. (1951).

Statistics were based on the Student's t-test.

RESULTS

Body weight

The body weight of the test animals was followed in tree groups. At the beginning the weight of the control animals was somewhat higher than in the test groups (Table 2). After a period of 100 days the mean weight of the animals varied from 39.7 ± 0.3 g to 40 ± 1.6 g. The weight of the controls had risen 12.9 g from the beginning of the experiment. In the test groups, T 1 and T 3 the weight had increased with 16 and 15.7 g, respectively.

Table 2. Weights of mice given a sigle intraperitoneal injection (5 mg) of titanium dioxide pigment. Mice were weighed before injection and 100 days later. The results are given in grams (mean ±S.E.).

Group	Weight at the beginning of the experiment	Weight after 100 days	Difference	N
Control (C_1)	26.8 ± 1.2	39.7 ± 0.3	12.9	5
Test 1 (T_1)	25.0 ± 0.8	41.0 ± 1.6	16.0	5
Test 3 (T_3)	24.8 ± 0.8	40.5 ± 2.0	15.7	5

Phagocytotic activity in vivo

In the first series of experiments the phagocytotic activity of mouse peritoneal cells was tested with titanium dioxide (T 1) and titanium dioxide pigment (T 3). In two days there was a prominent difference in the reaction to particles studied: under 10% phagocytes in the T 1, but more than 20% in the T 3 group (Fig. 1).

In the second series of experiments the phagocytotic activity of the pertitoneal cells was in two days 30-40% and thus higher than in the earlier studied T 1 and T 3 groups. After one week the amount of cells with ingested material increased in all groups but T 4 cells were clearly less actively phagocytizing than the T 2, T 5 and T 6 cells. Up to 15 days the number of phagocytes in the whole yield was again between 30 and 40% in all groups studied (Fig. 1).

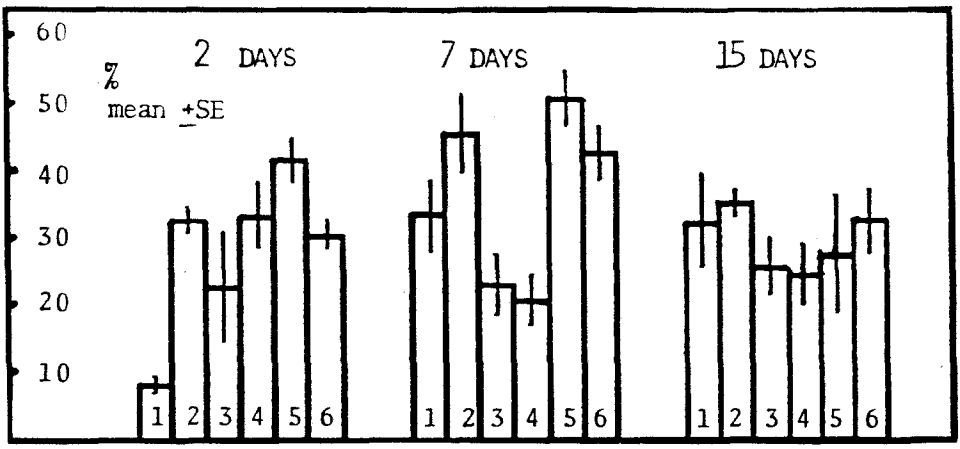

Figure 1. Phagocytosis of titanium particles by the murine peritoneal phagocytes in vivo. Numbers in bars = T1-T6.

Peritoneal cell count

The amount of cells counted in the lavaged exudate showed a rising trend from two to 15 days after intraperitoneal administration of the titanium pigments.

Acid phosphatase activity in the cells

The activity of acid phosphatase in the exudate peritoneal cells varied only slightly until the first day but on the third day the activity in the T 3 group reached a statistically highly significant ($p < 0.001$) peak that was about 70% greater than the control level. Also six days' exposure gave higher acid phosphatase activity in the T 3 cells than in the controls ($p < 0.01$, Table 3). The groups T 2, T 4, T 5 and T 6 had higher acid phosphatase activities than the control cells after one, three and six days exposure most of which being also statistically significant. The activities in the T 2 group were most prominent compared to the other groups. At every time period studied the activities of T 2 (anatase) were significantly higher ($p < 0.001$) than those of the control cells.

Table 3. Activity of acid phosphatase in mouse peritoneal phagocytes 1-6 days after the administration of titanium dioxide particles.

Group	1 day	3 days	6 days	N
Control 1	2.92 ± 0.17	4.21 ± 0.20	3.10 ± 0.06	5
T 1	3.44 ± 0.25	4.39 ± 0.23	3.22 ± 0.09	5
T 3	2.74 ± 0.22	7.23 ± 0.34***	3.95 ± 0.18*	5
Control 2	1.96 ± 0.18	3.11 ± 0.16	3.09 ± 0.17	8
T 2	2.99 ± 0.26***	5.45 ± 0.48***	5.02 ± 0.23***	8
T 4	2.88 ± 0.28*	4.07 ± 0.33*	3.83 ± 0.19*	8
T 5	2.57 ± 0.26**	4.40 ± 0.34**	3.60 ± 0.31	8
T 6	2.77 ± 0.21*	4.49 ± 0.62*	3.69 ± 0.24	8

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

β-glucuronidase activity in the cells

In the control group (C 1) the activity of β-glucuronidase was highest at all time intervals studied. In the T 1 group the activity of this hydrolytic enzyme was mostly at the control level but in the T 3 group the activity decreased (Table 4).

Table 4. Activity of β-glucuronidase in mouse peritoneal phagocytes 3-6 days after the administration of titanium dioxide particles.

Group	1 day	3 days	6 days	7 days	N
Control 1		5.38 ± 0.66	4.18 ± 0.31	5.18 ± 0.51	5
T 1		4.16 ± 0.49	3.89 ± 0.38	4.81 ± 0.43	5
T 3		4.92 ± 0.91	3.07 ± 0.72	2.18 ± 0.95*	5
Control 2	1.15 ± 0.21	2.00 ± 0.16	2.02 ± 0.13		8
T 2	1.37 ± 0.12	2.72 ± 0.26	3.54 ± 0.22***		8
T 4	1.01 ± 0.11	1.73 ± 0.35	2.49 ± 0.18		8
T 5	1.22 ± 0.18	1.70 ± 0.17	2.25 ± 0.50		8
T 6	1.22 ± 0.07	2.30 ± 0.22	2.41 ± 0.14		8

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

In the second series of experiments the activity of β-glucuronidase varied from 1.01 ± 0.11 nmol/µg protein in the T 4 cells to 1.37 ± 0.12 nmol in the T 2 cells. After three days of exposure the enzyme activity in T 2 cells had further increased and it was statistically highly significant ($p < 0.001$) six days from the intraperitoneal administration compared to control cells. In all groups studied the activities were close to control values.

DISCUSSION

The present study gave some hints of the possible toxicity of titanium dioxide and its pigments as to the variables used for comparative experimentation with silica rich and poor particles. The injection of titanium pigments shows a reaction of the whole organism as the mobilization of mononuclear phagocytes starts. The toxicity is, of course, partly dependent on the dose of the test material.

In this case, the TiO_2 pigments did not cause any acute poisonous effets. The body weight did not show any marked changes between the three groups studied.

Phagocytotic activity of the peritoneal cells revealed some differences between groups. The titanium dioxide (TiO_2, group T 1)gave a fairly low response two days after injection, but the silica rich pigment (T 3) reacted faster and did not change much during the whole experiment. In further studies (T 2, T 4, T 5 & T 6) the phagocytosis of pigment particles resulted in a higher uptake than in the T 1 and T 3 groups. The average amount of pigment loaded cells varied from about 25 to 50% in 2-15 days.

The intraperitoneal administration of titanium dioxide pigments to mice increased the cell number in the peritoneal cavity time dependently in the control (C 1) and the test groups (T 1, T 3). This shows a fairly low stimulation of the mononuclear cell system. The increased cell number may be resulted by cell

mortality which leads to the release of pigments back to the peritoneal cavity and stimulates the continuous mobilization of phagocytes to the irritated area of the body. In the cells the toxicity is dependent on the size of the particle as the area which reacts with the phagolysosome is greater in small particles. In the case of titanium pigments the size varies somewhat due to pigment quality.

The response of phagocyte acid phosphatase is not very clear according to the results of this study. On the other hand, this phenomenon is also seen in the T 2 group cells that have received almost pure titanium dioxide. In the other groups, there has also occurred some statistically significant changes but not at the same extent as in the two groups, T 3 and T 4. In the groups T 2 and T 3, the stimulation of acid phosphatase was highest after three days showing statistically highly significant ($p < 0.0001$) increase in activity compared to control cell activities.

The activity of β-glucuronidase in the peritoneal phagocytes did not show as much variation as the acid phosphatase in the same cells. The most exceptional group was again T 2 which had highest activities of all after a six days' interval. The changes were practically small in all other groups after one, three and six days.

The reaction of the peritoneal cell population to foreign material given as an injection to the cavity gave results that vary depending on the test method and are not correlative with each other. The silica rich pigment (T 3) gave some statistically differing results when compared to the other groups, especially in the acid phosphatase activity but, on the other hand, the response of β-glucuronidase was in this very case slight compared to many other groups. The titanium dioxide pigment (T 2) which has only small amounts of impurities as covering substance or for some other purpose, gave very interesting results in the enzyme tests conducted. As an average, the activities of β-glucuronidase were somewhat above the others. In the case of these enzyme activities, the inhibition seen in some of the groups may be due to the leakage of enzymes to the cavity that decreases the activities found in the cells. The T 2 group does not show any exceptional behavior in the phagocytotic activity or in the peritoneal exudate cell counts studied. ALLISON et al. (1966) have shown that p-nitrophenylphosphatase (here acid phosphatase) and β-glucuronidase are released to the culture medium, at least in vitro. This could explain the inhibition β-glucuronidase in the T 3 group but the result differs from the findings of acid phosphatase that gave statistically significant stimulation. Hence it seems that the leakage of enzymes is possible, if the stimulation of acid phosphatase is so high that the amount which could be released into the culture medium would not affect the enzyme quantitity inside the cells. The behavior of β-glucuronidase is not as easy to explain on the same basis because of different responses to the pigments injected.

ZITTING & SKYTTÄ (1979) also used anatase (T 2) as a test particle in studying the hemolytic effects on the human erythrocytes. They found disruption of the red cell membranes by using far higher concentrations than in this study. Anyhow, the mechanism might be at least analogous in case of the lysosomal membranes. Perhaps the action of anatase on the peritoneal cells is only stimulating and does not cause any harm to the lysosomes, although the synthesis of acid phosphatase and β-glucuronidase must have increased. Accordingly the reason for the exceptional activity of anatase in these tests is probably due to the structure and other physicochemical characteristics of the particle differing from the other test pigments used.

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