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Factors Involved in the Cytotoxicity of Kaolinite towards Macrophages *in Vitro*

by Reginald Davies*

The cytotoxicity of a high purity Cornish kaolinite toward mouse peritoneal macrophages *in vitro* was examined. The material was cytotoxic towards these cells, the activity could be decreased substantially by pretreating the dust with poly(2-vinylpyridine *N*-oxide). Pretreatment of the dusts with poly(acrylic acid) had a small effect on cytotoxicity, but combinations of the polymer treatments virtually abolished the material's biological activity towards macrophages. These studies indicated that the cytotoxicity of kaolinite is not due to its flakelike morphology.

Introduction

The prevalence of pneumoconiosis in the British china clay industry is under investigation at our Unit. Sheers (*1*) examined 553 Cornish china clay workers and found evidence of kaolinosis in 9%. Higher prevalences were found in the dustier occupations of milling, bagging and loading of china clay. Oldham (*2*) reporting on a radiological survey of 1676 men in the above industry found that 77.4% were within category 0, 17.9% in category 1, and 4.7% in categories 2 and 3 (*3*).

In animal studies, King et al. (*4*) found that intratracheal injection of kaolin into rats caused only a mild reticulin reaction not comparable with the fibrosis produced by quartz. Attygalle et al. (*5*) demonstrated that a combination of kaolin and dead tubercle bacilli caused a mainly collagenous reaction leading to well-marked fibrosis, the combined action producing more marked lesions in the lung than either material alone. The fibrogenicity of china clay is currently being examined in inhalation studies using specific pathogen-free Fischer rats at our unit.

Few *in vitro* studies have been carried out on kaolinite, the main component of china clay dust. Marks and Nagelschmidt (*6*) found that the material's toxicity towards guinea pig peritoneal macrophages was relatively low, a finding confirmed by Le Bouffant et al. (*7*). Recently, Low et al. (*8*) concluded that an extremely pure American kaolinite of respirable size was cytotoxic towards rabbit alveolar macrophages.

Electron micrographs of well-crystallized kaolinite show well-formed six-sided flakes often with a prominent elongation in one direction. The unit structure of kaolinite is composed of a single silica tetrahedral sheet and a single alumina octahedral sheet (Gibbsite sheet) combined in a unit so that the tips of the silica tetrahedrons and one of the layers of the octrahedral sheet form a common layer. The kaolinite particles might ultimately be expected to have a Gibbsitelike surface (aluminum hydroxide) and a silica surface (comprising siloxane Si-O-Si bonds); however, the particle surface is alleged to have an aluminosilicate "gel" coating rich in silica of unknown structure (*9*).

The net electrophoretic charge on kaolinite is negative, but at the edges of the flakes where the silica and alumina sheets are disrupted and primary bonds broken there is some evidence to suggest that the edge charge is positive (*10*).

In this study we examined the effect of poly (2-vinylpyridine *N*-oxide) (PVPNO), a well-known antagonist of quartz cytotoxicity (*11*), and the effect of poly(acrylic acid) on the *in vitro* cytotoxicity of kaolinite toward macrophages.

Methods

Culture of Macrophages with Dusts

Unstimulated mouse peritoneal macrophages were obtained by lavage of 22-27 g female T.O mice (Tuck and Son Ltd, Battlesbridge, Essex) with 3 mL Medium 199 (Flow Laboratories, Irvine, Scotland) containing 5 IU heparin, 100 U benzylpenicillin and 100 μg streptomycin/mL. Approximately 1.2×10^6 cells (in 2 cm^3 of the above medium) were added to each well of Linbro tissue culture multi-well plates

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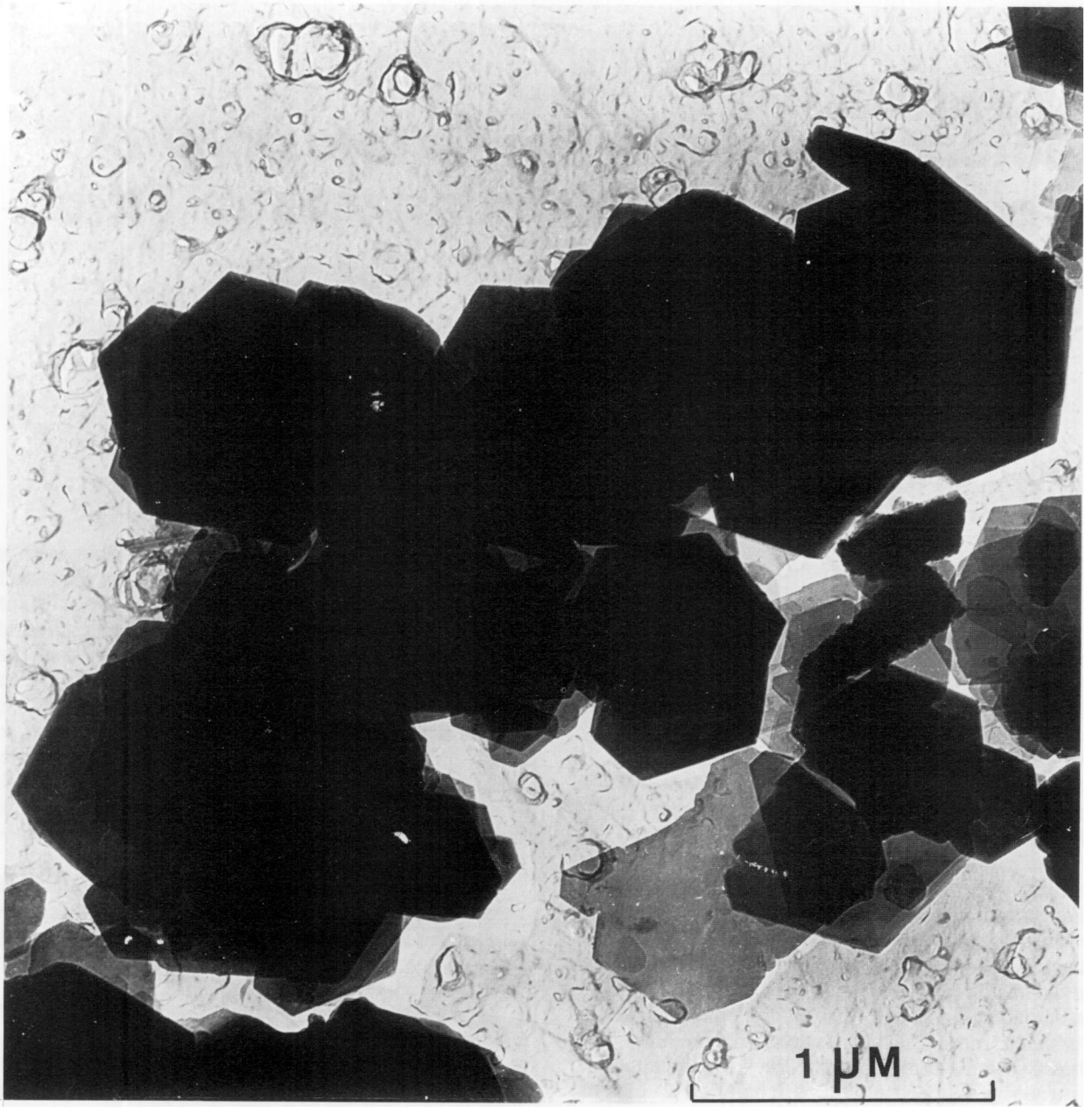

FIGURE 1. Electron micrograph of kaolinite.

(Flow Laboratories, Irvine, Scotland; well diameter 24 mm), and left for 1 hr at 37°C in a 5% CO_2/95% air atmosphere.

At the end of the period the nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and 2 mL Medium 199 containing the above antibiotics and 10% newborn calf serum (Flow Laboratories, Irvine, Scotland) was added. This serum had previously been heat-inactivated (56°C for 30 min) and acid-treated (*12*). Cultures were then left for 24 hr at 37°C in a 5% CO_2/95% air atmosphere before addition of fresh medium containing dusts.

Stock kaolinite solutions were made up in PBS at appropriate concentrations, ultrasonication being used to disperse the dust. The treatment of kaolinite with PVPNO or polyacrylic acid is described below. The kaolinite (treated or untreated) was added to the above medium containing 10% serum at a concentration of 40 $\mu g/cm^3$ and left for 2-3 hr at

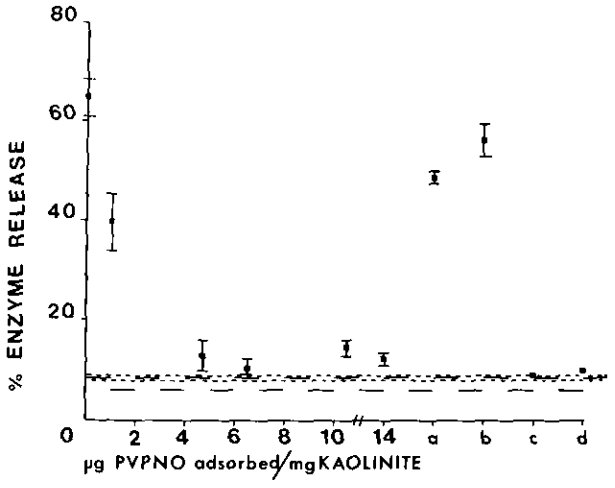

FIGURE 2. Effect of kaolinite on the release of lactate dehydrogenase from macrophages: (upper broken line) 40 $\mu g/cm^3$ magnetite, (lower line) no dust; (*a*, *b*) 0.025 and 0.1% PAA-treated kaolinite; (*c*) PAA treatment followed by PVPNO treatment; (*d*) PVPNO treatment followed by PAA treatment.

37°C before addition to the macrophage cultures. A 2 mL portion of "dusted" medium was added to each of four culture dishes which were then incubated for another 18 hr.

At the end of this period the medium was collected and the adherent cells disrupted by addition of 2 mL saline containing 0.1% Triton X-100 and 0.1% bovine serum albumin, and by rubbing the dish with a sterile siliconized rubber bung. Both medium and cell lysates were centrifuged at 500*g* for 10 min and the supernatants assayed for lactate dehydrogenase (LDH) by the continuous flow fluorimetric method of Morgan et al. (*13*) by use of a Perkin-Elmer Model 3000 fluorescence spectrometer.

The release of LDH from the cells into the culture medium is an indicator of dust cytotoxicity and is calculated as:

$$\text{\% enzyme released into culture medium} = 100\,[M/(M + C)]$$

where M is the enzyme activity of the medium and C is th enzyme activity of the cell lysate. The mean percent enzyme released for the four cultures used for each dust treatment, together with the 95% confidence intervals, was calculated.

Poly(2-vinylpyridine *N*-oxide) (PVPNO) and Poly(acrylic Acid) (PAA) Treatment of Dusts

PVPNO was synthesized according to the method of Gregson and Holt (*14*).

To sterile centrifuge tubes containing 10 mg kaolinite in 1 mL saline, 6 mL PBS containing 0-500 µg PVPNO was added. The mixture was ultrasonicated for 30 sec and allowed to stand at room temperature for 10 min. At the end of the period, the tubes were centrifuged at 3000 rpm and the supernatants removed. Unbound PVPNO was detected in the supernatant by measuring the optical density of the solutions at 260 nm using a Cecil CE5095 spectrophotometer. By using a calibrated curve the amount of PVPNO left unbound, and hence, by difference, the amount of PVPNO bound to the dust samples could be calculated.

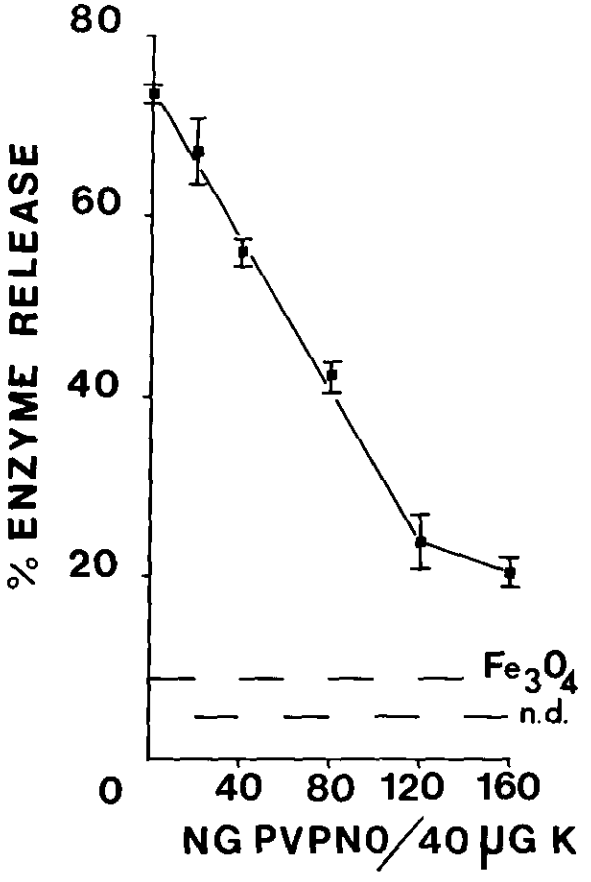

FIGURE 3. Effect of PVPNO treated kaolinite on lactate dehydrogenase release from macrophages: (Fe_3O_4) 40 $\mu g/cm^3$ magnetite; (n.d.) no dust.

Samples of kaolinite (10 mg in 1 mL PBS) were treated with 0.025% and 0.1% PAA in PBS, dispersed by ultrasonication and centrifuged as above to remove unbound PAA. One of the 0.1% PAA treated kaolinite samples was treated with 500 µg PVPNO as above (unbound PVPNO being removed as above). Another kaolinite sample was treated with 500 µg PVPNO (as above) and following removal of unbound PVPNO was treated with 0.1% PAA.

Results and Discussion

The kaolinite specimen (obtained from the St. Austell area of Cornwall) is shown in Figure 1 and contains 98% kaolinite and 2% mica as indicated by X-ray diffraction analysis. Of the material 98% was <5 μm in diameter.

Figure 2 shows that the material is cytotoxic toward mouse peritoneal macrophages, causing the release of considerable amounts of the cytoplasmic enzyme lactate dehydrogenase from the cells. In contrast, little enzyme release occurs when the macrophage cultures have been treated with a similar amount of magnetite—a nonfibrogenic dust (*15*)—or with no dust.

Figure 2 also shows that with increasing adsorption of PVPNO onto the kaolinite its cytotoxicity falls, but it does not fully reach the enzyme release level obtained with magnetite. Figure 3 shows that the cytotoxicity of the kaolinite falls linearly with increased adsorption of PVPNO, the amount of which needed to substantially lower the cytotoxicity (3μg/mg kaolinite) is significantly less than the amount of PVPNO which can be adsorbed on to the dust (Fig. 2). This may suggest that only some of the polymer binding sites are involved in the dust's cytotoxicity.

Figure 2 shows that the poly(acrylic acid) treatment of the kaolinite had a small effect on the dust's cytotoxicity. Although the actual binding of poly (acrylic acid) to the kaolinite was not demonstrated in this study, it has previously been shown by use of radioactively labeled poly(acrylic acid) (*16*). The binding of the polymer is likely to occur at the positively charged edges of the mineral, which suggests that the edges of the mineral particle only play a small role in the dust's cytotoxicity. Figure 2 also shows that when the kaolinite is treated both with PVPNO and PAA, its cytotoxicity reaches that of magnetite, i.e., the combination of the two polymers has eliminated the residual activity of the PVPNO-treated kaolinite. These studies indicate that the cytotoxicity of the kaolinite is not due to its flakelike morphology.

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