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Response of Bovine Alveolar Macrophages *in Vitro* to Welding Fume Particles

by Linda R. White,* Anne B. L. Marthinsen,* Karin Jakobsen* and Kristen B. Eik-Nes*

Alveolar macrophages (AM) from bovine lungs were exposed in culture to manual metal arc (MMA) welding fume particles, chromium (Cr), UICC chrysotile A or anatase for 17–20 hr. All the welding particle samples were more cytotoxic to AM than to anatase. Particles from the welding of mild steel with a rutile-coated electrode were less cytotoxic than those produced with a basic-coated electrode. Particles from the welding of stainless steel were slightly more cytotoxic, and much of this activity was probably due to CrVI. Selective release of *N*-acetyl-β-glucosaminidase (β-NAG) was only detected after exposure of AM to chrysotile. Supplementation of the incubation medium with 10% serum increased the viability of all exposed AM cultures, an effect not produced by serum albumin alone. Incubation of particle samples with dipalmitoyl phosphatidylcholine (DPPC) prior to addition to AM reduced the cytotoxicity of the "rutile" welding particles and of chrysotile.

Introduction

The manual metal arc (MMA) welding process produces a complex fume in which most of the particulate fraction is respirable (*1*) and of considerable compositional variety, depending on the process employed (*2-4*). Particles reaching the alveolar regions of the lung are likely to be engulfed by alveolar macrophages (AM). The cytotoxicity of certain mineral dusts *in vitro* and the possible association with pathological lung changes have been the subject of many studies (*5-9*), and several have employed AM in culture (*10, 11*). In this work, the effects of three different types of welding fume particles on bovine AM have been observed and compared with the activity of titanium dioxide (anatase) and UICC chrysotile A.

Materials and Methods

Test Substances

Welding particles were obtained from three sources: (1) from the welding of stainless steel with a rutile basic-coated electrode (hereafter referred to as "stainless steel particles"); and from the welding of mild steel with (2) a basic-coated electrode (hereafter "basic" particles) or (3) a rutile-coated electrode (hereafter "rutile" particles). The production and composition of the welding particles are described more fully elsewhere (*12, 13*). Titanium dioxide (99% anatase) was from BDH, Poole, Dorset, UK, and UICC chrysotile A asbestos was a gift from Dr. R. J. Richards, Cardiff, UK. All particulates were heat-sterilized, suspended in sterile 0.9% NaCl to a concentration of 1 mg/mL and sonicated (20 kHz, 100 W, 30 sec) prior to addition to the cell cultures. Chromium salt solutions [$K_2Cr_2O_7$ for Cr(VI), $CrCl_3 \cdot 6H_2O$ for Cr(III), both Merck] were made up to concentrations equivalent to Cr(VI) in the stainless steel particles and sterile-filtered.

Alveolar Macrophages (AM)

Bovine lungs were lavaged, essentially by the method of Myrvik et al. (*14*), with sterile 0.9% NaCl containing 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma) within 1 hr of animal slaughter. The cells were sedimented by centrifugation at 300*g* for 10 min and then washed three times in the sterile salt solution. Giemsa-stained smears were used to confirm at least 90% monocyte-type cells.

*Institute of Biophysics, NTH, University of Trondheim, N7034 Trondheim-NTH, Norway.

Populations contaminated by other cell types were discarded.

The washed cell pellet was resuspended in Eagle's MEM (Gibco Europe, Scotland) supplemented with 10% heat-inactivated (56°C, 30 min) newborn calf serum, and 1.5-2.0 × 10^6 cells were seeded into 5 cm diameter plastic Petri dishes (Nunc Products, Denmark) containing 3 mL culture medium and incubated at 37°C in 5% CO_2/95% air. After 1 hr the nonadherent cells were removed and 3 mL fresh medium added. After a further 3 hr, the medium was replaced with 5 mL medium ± 10% serum or 0.32% bovine serum albumin (BSA, Fraction V, Sigma) as required. Aliquots (0.05-0.5 mL) of the test substances were added to the desired concentrations. Samples containing dipalmitoyl phosphatidylcholine (DPPC, Sigma) were prepared by adding 0.1 mL DPPC dissolved in ethanol to particle suspensions, to a final concentration of 0.1 mg DPPC/mg particles, and incubating with agitation at 37°C for 30 min. Suspensions were dispersed vigorously before addition to cell cultures. Control cultures received 0.9% NaCl only. Incubation was then continued for a further 17-20 hr (time taken to process longest experiments).

Cell Studies

After 17 hr, AM populations contained both adherent and nonadherent cells. Cell number counting by hemacytometer and trypan blue exclusion (0.5% in balanced salt solution, BBS) were carried out on both populations. Enzyme levels were measured in both the cells and medium. Free cells were pelleted by centrifugation and then all cells lysed with 0.2% Triton X100 in Hanks' BSS. Lactate dehydrogenase (LDH) assays were carried out as described by Schnyder and Baggiolini (*15*) and *N*-acetyl-β-glucosaminidase (β-NAG) was assayed fluorimetrically (*16*) as described by Bowers (*17*). All experiments were carried out on at least two occasions in triplicate.

Results

At 17 hr after particle addition, control cultures in serum-free medium were found to contain both firmly attached cells requiring trypsin for removal and cells which were completely free in the medium or only loosely attached. These latter cells constituted 40-60% of total cells. Many attached cells had extensive membranes and sometimes fibroblastlike morphology. In welding fume particle-exposed cultures, cell detachment increased with dose. At the highest dose (40 μg/mL), about 95% of the cells were rounded and could be released from the dish by gentle agitation with a Pasteur pipet. Between 90 and 95% of all cells exposed to the various welding particulates contained fume particles at this dose. By comparison, cell morphology in anatase-exposed cultures resembled that of controls, even though 95% of the cells contained dust.

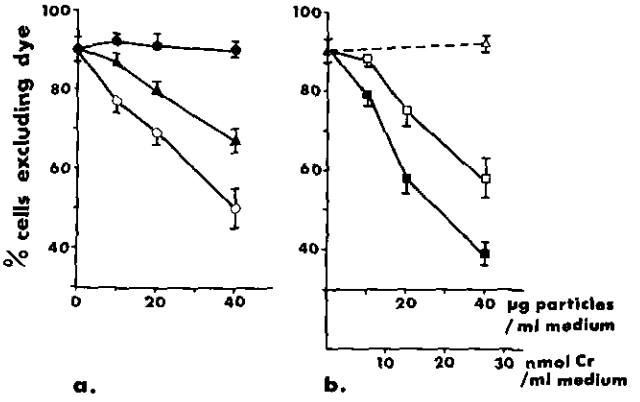

FIGURE 1. Trypan blue exclusion by bovine alveolar macrophages after exposure to welding particles, anatase or chromium: (●) TiO_2 (anatase); (▲) rutile welding particles; (○) basic welding particles; (△) Cr (III) (from $CrCl_3 \cdot 6H_2O$); (□) Cr (VI) (from $K_2Cr_2O_7$); (■) stainless steel welding particles. Values represent the mean ± SD.

Using trypan blue exclusion as a measure of cell viability, anatase was not cytotoxic up to a dose of 40 μg/mL (Fig. 1*a*). However, all welding fume particulates were active, the decrease in viability being roughly proportional to dose over the exposure range (Fig. 1). Cell counts of the various populations, however, did not indicate significant cell lysis, even at the highest dose. Rutile was the least toxic and stainless steel the most toxic of the welding particulates, though basic particles were almost as active as those from stainless steel welding. Cr(VI), in concentrations equivalent to those found in the stainless steel particles, was toxic to AM, but less so than the total particulate material. For comparison, Cr(III) had no effect on viability at these concentrations (Fig. 1*b*).

Enzyme release studies for LDH and β-NAG were found to parallel trypan blue exclusion for cultures exposed to anatase, and basic and rutile welding particles (Fig. 2*a-c*). However, there was some discrepancy between these parameters at the highest doses of Cr(VI) or stainless steel particles (Fig. 2*e, f*). In chrysotile-exposed cultures, there was consistency between trypan blue uptake and LDH release, but β-NAG release was significantly greater over the exposure range (Fig. 2*d*). Total β-NAG was not found to be significantly increased for any exposure group in comparison to controls, but total LDH was reduced by 10-15% for all exposure groups except anatase.

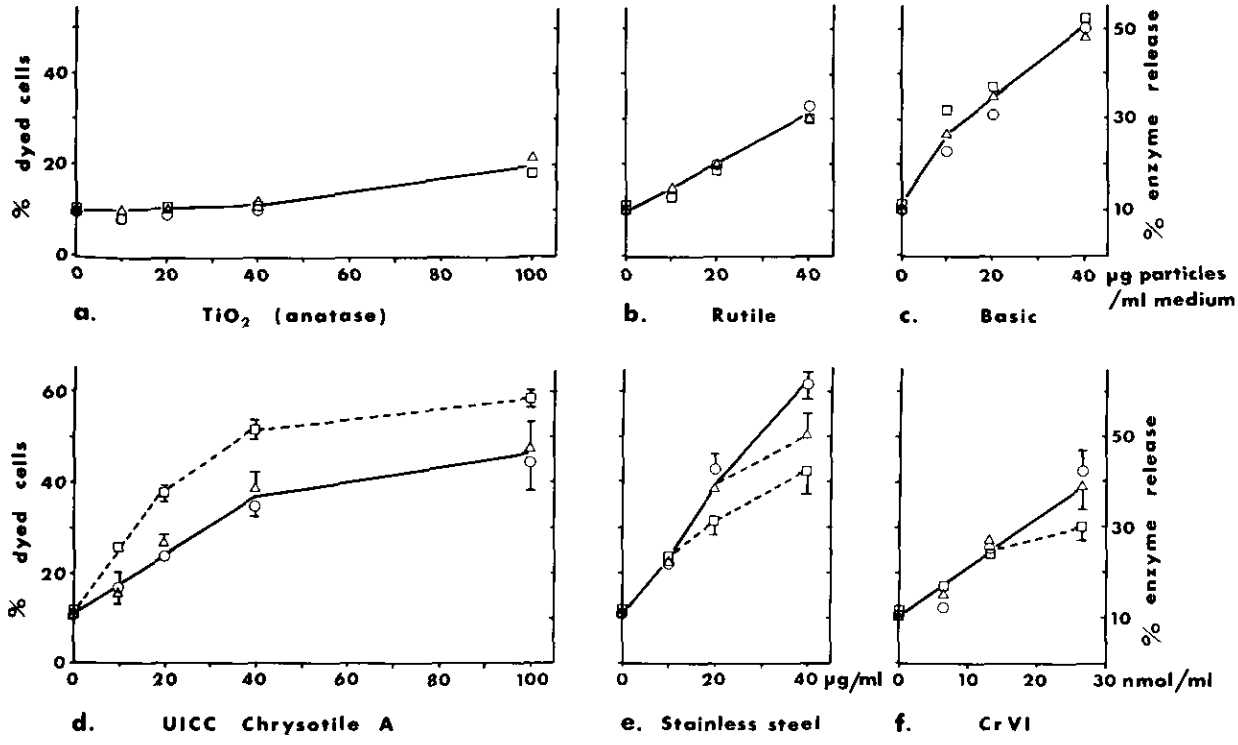

FIGURE 2. Comparison of trypan blue uptake, LDH and β-NAG release from bovine macrophages: (○) trypan blue uptake; (△) LDH release; (□) β-NAG release. For clarity, SD of the means are only shown where significant differences or wide deviations exist.

Supplementation of the culture medium with 10% newborn calf serum significantly reduced the toxicity of all exposure groups (Table 1). The various welding particulates maintained the same order of toxicity, but the activity of the stainless steel particles was almost the same as that of Cr(VI) alone. This improvement of cell viability effected by serum was not found when bovine serum albumin alone was added to the medium, and in fact the rutile welding particles were found to exert a slightly increased toxicity (Table 1), though no morphological difference was observed.

When the main constituent of pulmonary surfactant, DPPC, was added to the samples prior to addition to the cultures in serum-free medium, the welding particles had a tendency to aggregate. Consequently, results were not obtained for the basic particles, but better dispersion was obtained for the remaining samples after vigorous agitation. The presence of DPPC did not affect the activity of either the stainless steel particles or Cr(VI), but appeared to reduce the toxicity of both chrysotile and rutile welding particles (Table 1). Microscopic examination of AM after exposure to DPPC-treated chrysotile or rutile particles indicated that, while as many cells contained particles as in the absence of DPPC, the

Table 1. Effect of cytotoxicity of either 10% serum or 0.32% serum albumin addition to medium or DPPC treatment of particle suspension.

	% Dyed cells, serum free medium	LDH release, %			
		Serum-free medium	10% newborn calf serum	0.32% BSA	DPPC (0.1 mg/mg particles)
Control	10 ± 3	11 ± 3	7 ± 3	11 ± 1	11 ± 2
Chrysotile (40 μg/mL)	34 ± 2	38 ± 4	24 ± 0*	37 ± 7	21 ± 3*
Rutile (40 μg/mL)	33 ± 3	30 ± 1	16 ± 2*	41 ± 1*	18 ± 5*
Basic (40μg/mL)	50 ± 5	48 ± 4	21 ± 1*	51 ± 6	—
Stainless steel (40μg/mL)	61 ± 3	50 ± 5	34 ± 2*	57 ± 5	55 ± 2
Cr (VI) (27 μM)	42 ± 5	39 ± 6	28 ± 2*	48 ± 5	39 ± 2

*Significantly different from corresponding exposure in serum-free medium.

cell load appeared to be slightly less, especially in the case of the rutile particles. Particle aggregation was probably responsible for this. However, the morphology of cells exposed to DPPC-treated chrysotile or rutile resembled more that of control cultures (better cell attachment and spreading).

Discussion

The results show than in serum-free medium, all three welding fume particulates had greater cytotoxicity against AM than titanium dioxide, the activity increasing in the order, rutile<basic<stainless steel particles. Similar activity was found when these samples were instilled intratracheally into rat lung (*18*).

The main source of toxicity in the stainless steel particles appears to be Cr(VI), a conclusion also supported by results *in vivo* (*19*). While Cr(VI) is active against AM in micromolar concentrations, Cr(III) is nontoxic in this range, in agreement with the results of Waters et al. (*20*), who found Cr(III) toxic to cultured rabbit AM only at much higher concentrations.

Since AM number was not reduced after exposure to welding particles, it would seem that they cause membrane damage without cell destruction over the 17-20 hr exposure period. Measurement of extracellular LDH, a cytoplasmic enzyme, has also become a common marker for cell viability. Since it was discovered (*21*) that UICC chrysotile A could induce the selective release of lysosomal enzymes (such as β-NAG) from peritoneal macrophages, several studies have compared the relative release of LDH and lysosomal enzymes. Our results with chrysotile agree broadly with those, for example, of Jaurand et al. (*10*) and Morgan and Allison (*11*), derived from similar experiments with rabbit AM. However, in our system, while chrysotile induced a higher release of β-NAG than LDH, the asbestos was more cytotoxic than in most previous studies. Such differences probably result from differences in cell type and culture conditions. Selective release of β-NAG was not observed for any of the welding particulates, and release was in fact reduced at the highest concentrations of Cr(VI) and stainless steel particles in comparison to LDH. LDH release correlated well with trypan blue exclusion except, again, at these two latter exposures. It would seem that where cell viability is to be determined, some use of a dye-exclusion test should be made for comparison with enzyme release.

The increases in cell viability in all exposure groups when serum is added to the medium may have been due to cell/dust coating by serum components. Since BSA alone has no protective effect, the increased viability may simply be due to the richer culture conditions. In the case of Cr(VI) and stainless steel particles, it is also likely that serum factors will have reduced some Cr(VI) to Cr(III) (K. Jakobsen, unpublished results). The reason for the increased toxicity of rutile particles in BSA-supplemented medium is unclear.

Since dust particles reaching the alveolar spaces will become coated with pulmonary surfactant, the activity of these materials in the presence of the main component of the surfactant, DPPC, is interesting. However, the adsorption of DPPC to the particles has not been quantitated, and therefore the results are only preliminary. In addition, some particle aggregation was present, and cell particle concentration was assessed only by light microscopy. Nevertheless, the reduced toxicity of chrysotile in the presence of DPPC is in accordance with the results of Desai et al. (*22*), who found that coating chrysotile with surfactant inhibited chrysotile-induced erythrocyte hemolysis. In the case of rutile welding fume particles, the apparent reduction of toxicity after DPPC addition suggests that this may be a contributing factor to the comparatively low activity of this material *in vivo* (*13*).

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