BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 240–245 (1998)
ARTICLE NO. RC988405

Shedding of CD44 from PMA-Differentiated U-937 Cells Is Enhanced by Treatment with Mineral Particles

Noureddine Trabelsi, Anne Greffard, Jean Claude Pairon, Laurence Kheuang, Jean Bignon, Marie Claude Jaurand, and Yannick Pilatte[1]

INSERM Unité 139, Faculté de médecine, 8 rue Géneral Sarrail, 94010 Créteil Cedex, France, EA 2345 Université Paris XII

Received March 4, 1998

In this report, we show that enhanced shedding of CD44 might contribute to the down-regulation of this receptor observed after phagocytosis of MnO_2 particles by PMA-differentiated U-937. The apparent Mr of the soluble CD44 detected in culture supernatants was slightly lower than that of the membrane form suggesting that shedding resulted from proteolytic cleavage. Increased shedding of CD44 was also noted with other mineral particles (chrysotile and DQ12) but to a lower extent whereas some (TiO_2 and amosite) had no effect on this process. These results indicate that shedding enhancement was particle-specific rather than a general consequence of phagocytosis. The ability of the particles to enhance CD44 shedding was not directly dependent on their cytotoxic potency. Different patterns of reactivity were noted with CD11b, suggesting that the underlying mechanisms are specific.

Key Words: **Shedding; CD44; phagocytosis; U-937; mineral particles.**

The mechanisms regulating the expression of cell surface receptors are critical for the biology of the cell [1] and alterations of these mechanisms can lead to cell dysfunctions [1]. As for other proteins, expression of surface receptors can be controlled at several steps along the biosynthetic pathway leading from DNA to protein. Additional regulatory mechanisms of surface receptor expression can be involved when down-regulation is observed [2]. Receptors can be internalized by endocytosis and then either stored in intracellular vesicles or destroyed [2]. Alternatively, the extracellular domain of cell surface receptors can be proteolytically released into the extracellular space [3-5]. In the recent years, this shedding process has generated a great deal of interest and is now recognized as an important aspect of cell regulation [5]. However, despite its broad interest, in most cases the natural stimuli triggering shedding have still to be identified and the situations in which this process occurs remain to be determined [5-7]. In addition, the mechanisms leading to shedding of molecules from the cell surface are poorly understood [5-7].

Many of the receptors susceptible to be shed from the cell surface are involved in cell-cell interactions suggesting that shedding may be an important mechanism in the regulation of cell adhesion [5, 8, 9]. In this context, significant attention has focused on the hyaluronan receptor CD44, a molecule not only involved in cell adhesion but also in multiple other cellular functions [10, 11]. Shedding of CD44 was recently shown to be responsible for the down-regulation of this molecule on granulocytes upon activation [8] and was proposed to be essential for proper timing of CD44 participation in adhesion and other cellular functions [8, 9, 11, 12].

In a previous study we showed that phagocytosis of MnO_2 particles was accompanied by a significant reduction in the level of CD44 (Trabelsi et al. submitted) in both human alveolar macrophage and PMA-differentiated U-937 cells. In the present study we show that MnO_2 particles increase shedding of CD44 in PMA-differentiated U-937, suggesting that this process might contribute to the observed down-regulation. Other mineral particles were found to increase CD44 shedding but less efficiently than MnO_2. This effect was observed in a particle-specific manner rather than

[1] Corresponding author: INSERM U139, Faculté de médecine Paris XII, 8, rue du Général Sarrail, 94010 Créteil cedex, France. Fax: 33 1 49 81 35 33. E-mail: pilatte@im3.inserm.fr.

Abbreviations: Amo: Amosite, Chr: Chrysotile, DQ12: crystalline silica, MnO_2: manganese dioxide, TiO_2: titanium dioxide, EDTA: ethylenediaminetetraacetic acid, MTT: 3-(5,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, NPGB: p-nitrophenyl-p′-guanidobenzoate, PBS: phosphate buffered saline, PMA: phorbol myristate acetate.

0006-291X/98 $25.00

as a general consequence of phagocytosis and was not related to the cytotoxic potency of the particles. In addition, different patterns of reactivity were noted when other receptors were investigated, suggesting that the underlying mechanisms are specific.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium, glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from GibcoBRL (Cergy Pontoise, France). Mouse monoclonal antibody to human CD44 H (clone 2C5) was obtained from R&D Systems (Abingdon, UK), mouse monoclonal antibody to CD14 (clone MO2) from Coulter (Marseille, France) and mouse monoclonal antibody to CD11b from DAKO (Trappes, France). All chemicals were of analytical grade and were purchased from Sigma Chemicals (L'Isle d'Abeau Chesnes, France) unless otherwise stated.

Mineral particles and fibers. Crystalline silica (quartz DQ12), manganese dioxide (MnO_2), and titanium dioxide (TiO_2) were kindly given by Pr. D Lison (UCL, Brussels, Belgium). The asbestos fibers, Rhodesian chrysotile and amosite were provided by the Union Internationale contre le cancer (UICC).

Cell culture. U-937 cells (American Tissue Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2mM glutamine, 50U/ml penicillin and 50μg/ml streptomycin (designated as complete medium). In the experiments described herein, U-937 cells were differentiated with 0.162 μM of PMA during four days as previously described by Öberg et al. [13]. Cells were seeded either in 96 wells tissue culture plates at 5×10^4 cells /well to assess cell viability by the MTT assay or in 75 cm^2 tissue culture flask (10×10^6 cells/flask in 20ml complete medium) to assess CD44 shedding. After four days of PMA-differentiation, U-937 cells (approximately 30×10^6 cells/75 cm^2 flask) were extensively washed with sterile PBS and then exposed for 48h to several concentrations of the various particles or fibers as described below.

Preparation of particles and fibers and cell treatment. Particles and fibers were heated for 2 hours at 200°C to inactivate bacterial endotoxins. They were then suspended in complete medium and dispersed by sonication (50khz, 20W) for 5 min. An appropriate volume of particle or fiber suspension was then added to differentiated U-937. In order to achieve a final concentration of 50μg of particles per cm^2 of culture surface area, 20 ml of particle suspension at 187.5 μg/ml was added to each 75 cm^2 flask and 100μl of particle suspension at 160μg/ml was added to each well of 96 wells plates. After 48hr of incubation, the cells were subjected to the different analyses.

Viability test. The viability of differentiated U-937 cells exposed to the different particles was assessed by the MTT assay [14] as described elsewhere [15].

Preparation of cell lysates. After particle or fiber treatment in 75 cm^2 tissue culture flask, cells were washed twice with PBS, scrapped and adjusted to an approximate concentration of 10^7 cells/ml in ice cold homogenization buffer (PBS supplemented with 1% Nonidet P40, 25μM of the serine protease NPGB and 10mM EDTA). The suspension was homogenized using a glass Teflon homogenizer with 10 strokes and the resulting homogenate was cleared by centrifugation at 14,000 $\times$ g for 15 min. The protein concentrations was estimated by a procedure adapted from the Lowry assay [16], the BIO-RAD *DC* protein assay (Bio-Rad laboratories, Ivry sur Seine, France), as recommended by the manufacturer using bovine serum albumin as standard.

Concentration of cell culture media. After treatment with particle or fibers, culture supernatants were collected and centrifuged at 1,000 $\times$ g to remove floating cells. Each supernatant was brought to 80% saturation by adding appropriate amount of liquid saturated ammonium sulfate. After one hour under stirring at 4°C, the precipitated proteins were pelleted by centrifugation and resolubilised in a volume of PBS (500μl) sufficient to achieve a 40 fold concentration. Concentrated culture media were then extensively dialyzed against PBS and subjected to Western-blot analysis.

Western blotting. Cell lysates (10 μg of protein for each sample) or concentrated cell supernatants (20μl of concentrated supernatant for each sample) were first separated as described by Laemmli [17] on 10% polyacrylamide discontinuous mini slab gels in reducing conditions. Separated proteins were then electrophoretically transferred onto Immobilon membrane (Millipore Corporation Bedford, MA, USA) as described elsewhere [18]. After transfer, each membrane was probed with a specific mouse monoclonal antibody. Antibodies specific to CD11b and CD14 antigens were used at a concentration of 0.1μg/ml whereas anti CD44 was used at 0.5μg/ml. After extensive washing, the membranes were incubated appropriately with peroxydase-conjugated anti mouse IgG (Calbiochem, Meudon, France). The labeled proteins were visualized using Hyperfilm-ECL and ECL Western blotting detection kit (Amersham Labs, Little Chalfond, UK) according to the manufacturer recommendations. Proteins were quantified by scanning densitometry on a ScanJet IIC densitometer (Hewlett Packard) using Scan Analysis (version 2.0, Biosoft, Cambridge, UK).

Statistical analysis. Statistical analyses were performed using Student's-test and the Mann Whitney-U test. An alpha level(P) less than 0.05 was considered significant.

RESULTS

Upon differentiation, U-937 cells acquire the ability to phagocytoze mineral particles and they constitute a suitable model for investigating human alveolar macrophage response to mineral particles. The particles and fibers used in this study were previously found to ellicit differential fibrogenic or inflammatory responses. DQ12 and asbestos fibers cause a chronic inflammation that can ultimately lead to lung fibrosis [19]; MnO_2 particles induce a strong acute inflammatory process [20, 21] and TiO_2 is regarded as a harmless particle [22].

Effect of Mineral Particles on CD44 Expression

PMA-differentiated U-937 cells were incubated during 48h with various mineral particles at 50μg/cm^2. After treatment, CD44 expression was evaluated in cell extracts by western blotting. The typical experiment depicted in Fig. 1 confirmed that treatment with certain particles was accompanied by a marked decreased in CD44 expression and the more drastic effect was observed following treatment with MnO_2 particles. Densitometric analysis of the Western blot shown in figure 1 indicated that CD44 expression was reduced by nearly 80 % in MnO_2-treated cells whereas moderate reductions of about 4 and 12 % were found following treatment with DQ12 and TiO_2 particles respectively. Intermediate effects were observed with chrysotile or amosite as a 30-35 % decrease in CD44 expression was

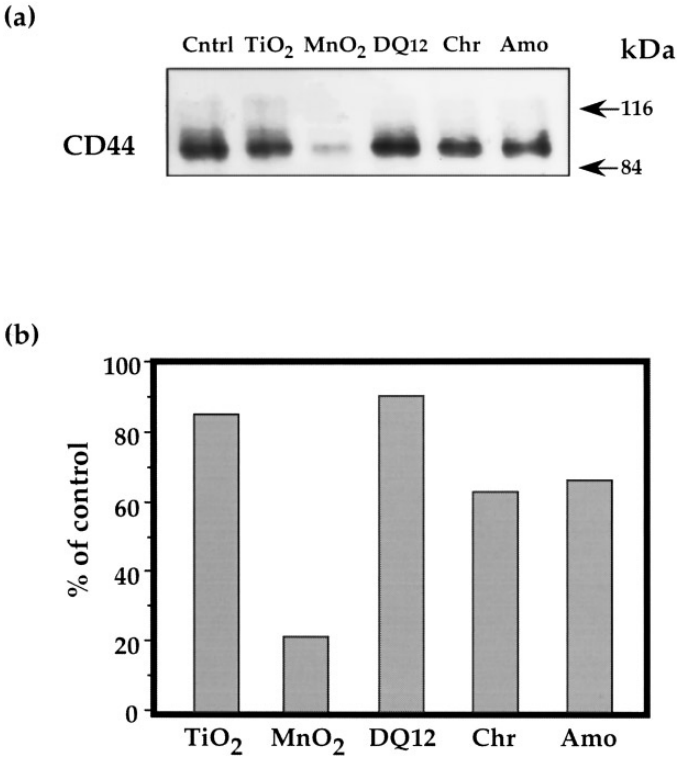

FIG. 1. Effect of mineral particles and fibers on CD44 expression in PMA-differentiated U-937. (**a**) Cells were treated with the various particles or fibers at 50μg/cm^2 for 48 hours. The cell lysates were run on SDS-PAGE (10μg of protein/lane) and CD44 is visualized by immunoblotting with a specific mouse monoclonal antibody. (**b**) Densitometric analysis of the western blot shown in panel **a.** The results are expressed as percentage relative to untreated control. Cntrl: control untreated cells, TiO_2: titanium dioxide, MnO_2: manganese dioxide, DQ12: crystalline silica, Chr: Chrysotile, Amo: Amosite.

detected in cells treated with either of these asbestos fibers. Thus with respect to their potency to reduce CD44 expression the mineral particles rank as follows: $MnO_2 >$ chrysotile $\approx$ amosite $>$ DQ12 $\approx TiO_2$.

Effect of Mineral Particles on Shedding of CD44 and Other Receptors

To determine whether shedding could contribute to the reduced expression of CD44 induced by mineral particles we investigated the putative presence of soluble CD44 in culture supernatants from treated cells. PMA-differentiated cells were treated for 48 hours with the various particles as described above. Cell culture supernatants were collected, concentrated 40 fold and subjected to western-blotting analysis for the detection of CD44. We show in figure 2a, that little amount of CD44 was spontaneously shed from untreated cells. This soluble form was effectively released from the cells as it was initially undetectable in the complete culture medium (not shown). CD44 shedding was significantly increased by treatment with some of the particles (Fig. 2a). Quantitation of three different experiments indicated that the amount of soluble CD44 detected in the media of MnO_2-treated U-937 was roughly 3 fold the amount found in the untreated control (Fig. 2b). Shedding of CD44 was also markedly enhanced by treatment with DQ12 or chrysotile since the level of soluble was 1.7 fold that of untreated cells following treatment with either particles (Fig. 2b). No significant changes were noted with either TiO_2 or amosite. Therefore, on a per weight basis, the potency of mineral particles to enhance CD44 shedding was in the following relative order: $MnO_2 >$ chrysotile $\approx$ DQ12 $>$ amosite $\approx TiO_2$. Noteworthingly, the apparent Mr of the soluble CD44 detected in culture supernatants was slightly lower compared to membrane CD44 (Fig. 2a) suggesting that the soluble form was released by proteolytic cleavage.

For comparison, the influence of mineral particles on shedding of other receptors was investigated. As shown in Fig. 3, the amount of soluble CD11b, the α chain of Mac-1 the C3bi receptor was markedly increased by treatment with DQ12, chrysotile or amosite whereas MnO_2 had only a moderate effect. It should be mentioned that detection of soluble CD11b required much longer exposure to hyperfilm ECL than that of soluble CD44 (120 versus 5 min.), suggesting that it was shed at a lower rate than CD44.

Shedding of CD14, the LPS receptor, was also investigated. No soluble form of this receptor was detected in the supernatants of control or particles-treated cells even when Hyperfilm ECL were exposed for an extended period (up to 5 hours).

Effect of Mineral Particles on Cell Viability

Phagocytosis of toxic mineral particles can induce a cell injury entailing a leakage of proteolytic enzymes which could contribute to the shedding process. Therefore it was of importance to determine whether the ability of mineral particles to enhance shedding of surface proteins was related to their cytotoxic potency. The effect of the various particles on the viability of differentiated U-937 was determined by the MTT assay. As shown in Fig. 4, the most cytotoxic particle was chrysotile which decreased cell viability by about 60 % whereas TiO_2 was almost harmless. The potency of the mineral particles to reduce cell viability was in the following relative order: chrysotile $<$ amosite $\approx$ $MnO_2 >$ DQ12 $> TiO_2$.

DISCUSSION

Despite the potential physiopathological importance of shedding, most frequently the natural stimuli triggering this process remain to be determined [5, 8]. Here we show that uptake of mineral particles such as

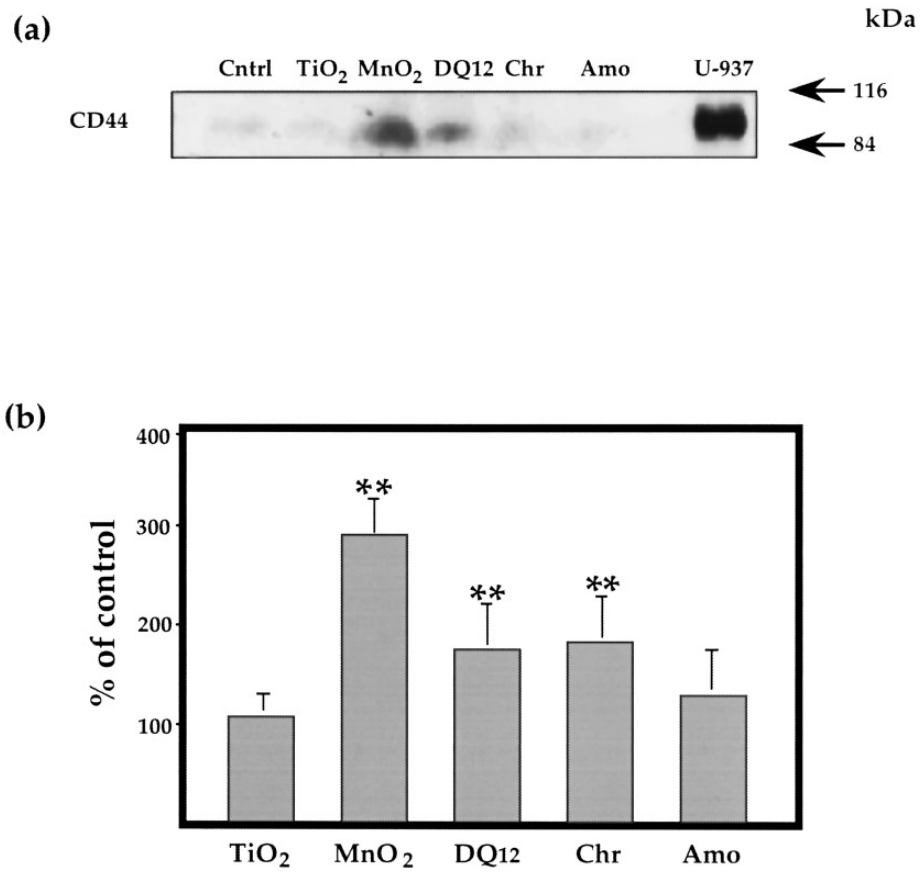

FIG. 2. Effect of mineral particles and fibers on CD44 shedding. (**a**). Cells were treated with the various particles or fibers at 50μg/cm^2 for 48 hours. The culture supernatants were collected, concentrated 40 fold and dialyzed. Concentrated media (20μl/lane) were run on SDS-PAGE and CD44 is visualized by immunoblotting with a mouse monoclonal antibody to human CD44H. A protein extract (U-937) from untreated differentiated cells was run for comparison (10μg of protein). This figure is one representative out of three experiments. (**b**) This histogram represents the densitometric analysis of three different experiments. The results are expressed as percentage relative to untreated controls. Bars represent the mean and the vertical brackets show 1 SE. **: $p<0.01$. See Fig. 1 for abbreviations.

MnO_2, DQ12 or chrysotile by PMA-differentiated U-937 cells is accompanied by a marked increase in CD44 shedding and in the case of MnO_2, this process is likely involved in the down-regulation of CD44 induced by this type of particle. Although all particles tested here were readily phagocytozed by the cells, certain such as TiO_2 or amosite had little effect on CD44 shedding indicating that phagocytosis *per se* is not sufficient to enhance CD44 release. MnO_2 which was the most cytotoxic particle was also the most efficient to enhance CD44 shedding whereas TiO_2 which is almost harmless did not influence this process. However, no relationship between cytoxicity and CD44 shedding was found with the other particles and the lack of correlation between shedding and cytotoxicity was confirmed by the observation that MnO_2 had little effect on shedding of CD11b.

The mechanisms for cell surface protein shedding are still unclear [4, 5]. Recent reports provided evidence for the existence of a single common mechanism leading to the release of a wide variety of surface proteins [6, 7] while other suggested that specific mechanisms may be involved in the shedding of different molecules [8, 9]. Our observation that MnO_2 enhanced CD44 shedding without affecting CD11b whereas amosite exerted opposite effects, indicates that these mineral particles influence shedding through specific mechanisms.

Limited proteolysis by metalloproteinases at a site close to the extracellular face of the plasma membrane appears likely in the generation of soluble CD44 [4-8]. This assumption is supported by the results presented here, suggesting that signals induced by the mineral particles that were found to markedly enhance CD44 shedding may up-regulate specific proteases involved in this process. Additionally, there is convincing evidence that conformational alterations of CD44 rendering the cleavage site more accessible to the putative protease(s) may be required for effective release [5, 8]. Such conformational changes can be induced by interaction of CD44 with its natural ligand [8] and can be

simulated by using monoclonal antibody to CD44 [8, 9]. Processes taking place at the cell-particles interface that are determined by the reactivity of the particle surface may lead to protein denaturation [23]. It is, therefore, tempting to speculate that in the early stage of phagocytosis when mineral particles such as MnO_2, DQ12 or chrysotile are in close contact with the plasma membrane, they interact with CD44 and induce conformational alterations of this molecule susceptible to promote shedding. Alternatively, recent observations suggest that changes in glycosylation may induce conformational alterations of CD44 which result in a significant increase in spontaneous shedding of this molecule [24]. In this respect, we have recently shown that DQ12 and MnO_2 which both increased CD44 shedding, induced specific pattern of alterations in the carbohydrate moieties of PMA-differentiated U-937 glycoproteins, whereas TiO_2 had little effect if any on this parameter [25]. Therefore, although CD44 was not specifically investigated in this study [25], it is conceivable that at least MnO_2 and DQ12 might increase CD44 shedding through a glycosylation-dependent mechanism.

In conclusion, increased shedding appeared to contribute significantly to the down-regulation of CD44 induced by MnO_2. As CD44 has been shown to be involved in the mechanisms maintaining and amplifying the inflammatory response [26, 27], shedding of this receptor may represent a specific aspect of macrophage response to this type of particle.

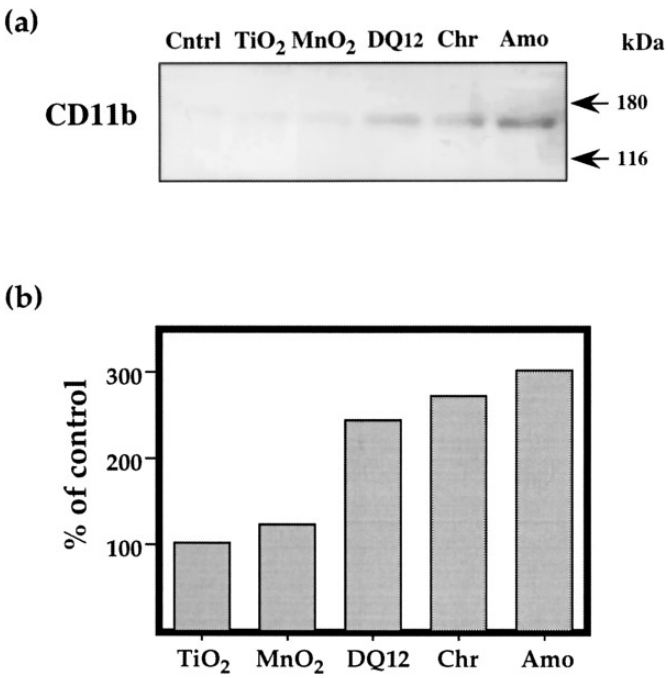

FIG. 3. Effect of mineral particles and fibers on CD11b shedding. (**a**). Cells were treated with the various particles or fibers at 50μg/cm^2 for 48 hours. The culture supernatants were collected, concentrated 40 fold and dialyzed. Concentrated media (20μl/lane) were run on SDS-PAGE and CD11b is visualized by immunoblotting with a specific mouse monoclonal antibody. (**b**) Densitometric analysis of the western blot shown in panel **a.** The results are expressed as percentage relative to untreated control. See Fig. 1 for abbreviations.

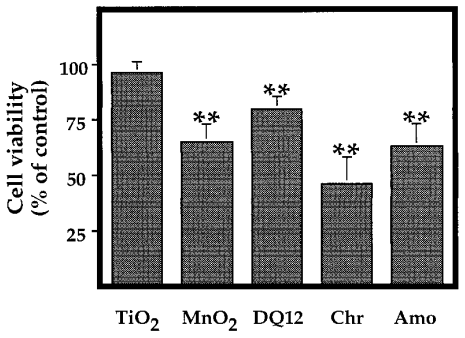

FIG. 4. Effect of particles and fibers on PMA-differentiated U-937 viability. Cells were treated with the various particles or fibers at 50μg/cm^2 for 48 hours and then the MTT assay was performed. The results are expressed as percentage relative to untreated controls. Bars represent the mean and the vertical brackets show 1 SE. **: $p<0.01$. See Fig. 1 for abbreviations.

ACKNOWLEDGMENTS

This study was supported by institutional funding from Institut National de la Santé et de la Recherche Medicale (INSERM) and by European Union grant EV5V-CT94-0399. The authors thank Pr. D Lison (UCL, Brussels) for providing DQ12, TiO_2 and MnO_2 particles and C. Vaslin for secretarial assistance.

REFERENCES

1. Darnell, J., Lodish, H., and Baltimore, D. (1986) Molecular Cell Biology. Scientific American Books, Inc.
2. Grady E. F., Böhm S. K., and Bunnett N. W. (1997) *Am. J. Physiol.* **273,** G586–G601.
3. Massagué, J., and Pandiella, A. (1993) *Annu. Rev. Biochem.* **62,** 515–541.
4. Rose-John, S., and Heinrich, P. C. (1994) *Biochem. J.* **300,** 281–290.
5. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) *Biochem. J.* **321,** 265–279.
6. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massagué, J. (1996) *J. Biol. Chem.* **271,** 11376–11382.
7. Müllberg, J., Rauch, C. T., Wolfson, M. F., Castner, B., Fitzner, J. N., Otten-Evans, C. Mohler, K. M., Cosman, D., and Black, R. A. (1997) *FEBS Letters* **401,** 235–238.
8. Bazil, V., and Horejsi, V. (1992) *J. Immunol.* **149,** 747–753.
9. Bazil, V., and Strominger, J. (1994) *J. Immunol.* **152,** 1314–1322.
10. Kincade, P. W., Zheng, Z., Katoh, S., and Hanson, L. (1997) *Curr. Opin. Cell Biol.* **9,** 635–642.
11. Naor, D., Sionov, R. V., and Ish-Shalom, D. (1997) *Adv. Cancer Res.* **71,** 241–319.

12. Katoh, S., McCarthy, J. B., and Kincade, P. W. (1994) *J. Immunol.* **153,** 3440–3449.
13. Öberg, F., Botling, J., and Nilsson, K. (1993) *J. Immunol.* **150,** 3487–3495.
14. Mossmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
15. Dong, H. Y., Saint-Etienne, L., Renier, A., Billon Galland, M. A., and Brochard, P. (1994) *Fund. Appl. Toxicol.* **22,** 178–185.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
18. Towbin. H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
19. Harvey, B. G., and Crystal, R. G. (1997) *In* R. G. Crystal, and J. B. West, et al. editors. The Lung: Scientific Foundations, 2nd Ed. New York: Raven Press, Ltd., 2339–2352.
20. Bergström, R. (1977) *Scand. J. Work Environ. Health* **3** Suppl. 1, 1–41.
21. Snella, M. C. (1985) *Toxicology* **34,** 153–159.
22. Nolan, R. P., Langer, A. M., Weisman, I., and Herson, G. B. (1987) *Brit. J. Indust. Med.* **44,** 687–698.
23. Fubini, B. (1997) *Environ. Health Persp.* **105**(Suppl 5), 1013–1020.
24. Bartolazzi, A., Nocks, A., Aruffo, A. Spring, F., and Stamenkovic, I. (1996) *J. Cell Biol.* **132,** 1199–1208.
25. Trabelsi, N., Greffard, A., Pairon, J. C., Bignon, J., Zanetti, G., Fubini, B., and Pilatte, Y. (1997) *Environ. Health Persp.* **105**(Suppl 5), 1153–1158.
26. Aruffo, A. 1996. CD44: One ligand, two functions. *J. Clin. Invest.* **98,** 2191–2192.
27. McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C., and Noble, P. W. (1996) *J. Clin. Invest.* **98,** 2403–2413.